Interleukin (IL)-23 Suppresses IL-10 in Inflammatory Bowel Disease

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Background: The deregulation of immune responses plays a critical role in inflammatory bowel disease (IBD).

Results: The levels of IgA and IL-10 were significantly lower, whereas the levels of IL-23 were higher, in IBD specimens.

Conclusion: IL-23 can suppress IL-10 in the IBD intestinal mucosa.

Significance: IL-23 may be a potential target in the treatment of IBD.

Interleukin (IL)-10 plays an important role in immune regulation in the intestine. Immune deregulation is suggested in the pathogenesis of inflammatory bowel disease (IBD). This study aims to elucidate the role of IL-23 in the suppression of IL-10 in the IBD intestinal mucosa. Surgically removed colon specimens were obtained from 16 IBD patients. The expressions of IL-10, IL-23, and IgA in the specimens were examined at the protein and gene transcriptional levels. The gene transcription of IL-10 was assessed by chromatin immunoprecipitation assay and promoter accessibility assay. The levels of IgA and IL-10 were significantly lower, whereas the levels of IL-23 were higher, in IBD specimens than in normal controls. The levels of IgA and IL-10 were negatively correlated with the infiltration of inflammatory cells in the IBD mucosa. The production of IL-10 by lamina propria mononuclear cells was lower in the IBD group than in the control group, and these levels could be enhanced by blocking IL-23. The gene transcription of IL-10 was significantly suppressed in CD4+ T cells of IBD mucosa; this phenomenon could be replicated in vitro by adding IL-23 to the culture of polarized Th2 cells. Overexpression of IL-23 in the intestinal mucosa suppresses the production of IL-10, which weakens the defensive barrier by reducing the production of IgA in the gut.

It is suggested that immune deregulation in the intestine plays a critical role in the pathogenesis of IBD. Crohn disease (CD) is mainly associated with a T helper (Th) 1 response (1, 2).

Ulcerative colitis (UC) is associated with Th2 response (2, 3). Tumor necrosis factor (TNF)α is found to play an important role in the pathogenesis of IBD based on the fact that administration of anti-TNFα can significantly ameliorate the pathogenesis of IBD (4). Recent advances also indicate that interleukin (IL)-23, mainly produced by macrophages, is one of the critical cytokines in IBD and is essential for promoting chronic intestinal inflammation (5, 6). IL-23 is constituted by the specific p19 subunit and the IL-12/p40 subunit (7, 8) (“IL-23p19” is referred to as “IL-23” throughout). The skewed elevation of IL-23 can induce the overproduction of IL-17 and IFN-γ (9).

A plausible etiology of IBD is that the intestinal immune system inappropriately attacks the commensal bacteria in the gut (10). Upon the invasion of bacteria, the first response is the infiltration of neutrophils and mononuclear cells in local tissue. Following the accumulation of neutrophils and mononuclear cells, the levels of myeloperoxidase (MPO) in the local tissue are increased (11). MPO is also one of the parameters in IBD modeling studies (12). Besides MPO, neutrophils and mononuclear cells contain several other chemical mediators and a series of cytokines that can contribute to the initiation of inflammation in the local tissue. Although studies on the pathogenesis of IBD have advanced rapidly in the last decade, the etiology of how the inflammation in the intestinal mucosa is initiated in IBD remains to be further understood.

Immunoglobulin (Ig) A is one of the five Igs, including IgA, IgD, IgE, IgG, and IgM, in the body. IgA makes up more than 75% of Igs (13). Upon exposure to microbiota in the intestine, abundant IgA-secreting plasma cells can be generated (14). One of the major functions of IgA is defense against microbial infections (15). High affinity IgA neutralizes microbial toxins and pathogens in addition to recognizing commensal bacteria (16). The significance of IgA in the maintenance of intestinal homeostasis is recognized, although the precise function of IgA in the prevention of bacterial infection still has to be further elucidated (15).

It is proposed that despite the wide variety of causative factors, intestinal mucosal inflammation can be induced by either an excessive Th1 cell response, which is associated with increased secretion of IL-12, IFN-γ, and/or TNFα or an excessive Th2 cell response, which is associated with increased secre-
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TABLE 1
Demographic data of IBD patients

| Group      | CD       | UC       |
|------------|----------|----------|
| Sex        | Male: 4; female: 3 | Male: 6; female: 3 |
| Age        | 55.5 (45–64) | 52.4 (49–64) |
| Weight (kg) | 60.9 (51–65) | 64 (52–69) |
| Duration of IBD (months) | 36 (26–59) | 39 (28–66) |
| Race       | Asian | Asian |
| Stenosis   | 7 | 0 |
| Perforation | 0 | 9 |

In this study, we observed that the level of IL-10 was negatively correlated with the infiltration of inflammatory cells in the colon mucosa of patients with advanced stages of IBD; IL-10 was an important facilitator in the production of IL-23 in the colon mucosa. Via the epigenetic assay, we found that IL-23 played a critical role in suppression of the gene transcription of IL10 in the IBD colon and in polarized CD4+ T cells.

MATERIALS AND METHODS

Patients and Collection of Colon Specimens—In this study, we collected surgically removed colon tissue from 16 IBD patients (nine UC patients with colon stenosis; seven CD patients with colon perforation; Table 1) and nine colon cancer patients (supplemental Table S1) at the China PLA General Hospital and at the affiliated hospitals of Tongji University and Zhengzhou University. The diagnosis of IBD was made on the basis of the endoscopic, radiological, histological, and clinical criteria provided by the World Health Organization (WHO) Council for International Organizations of Medical Sciences and the International Organization for the Study of Inflammatory Bowel Disease (19, 20). Written informed consent was obtained from each patient. The study using human specimens for research was approved by the Human Study Ethics Committees at Tongji University, Zhengzhou University, and China PLA General Hospital.

Lamina Propria Mononuclear Cell (LPMC) Isolation—Surgically removed tissue was collected, cut into about 2 × 2 × 2 mm, and treated with predigestion solution (1× Hanks’ balanced salt solution containing 5 mM EDTA and 1 mM DTT) at 37 °C for 30 min under slow rotation. After centrifugation (×1000 rpm, 10 min), the samples were incubated in the digestion solution (0.05 g of collagenase D, 0.05 g of DNase I, and 0.3 g of dispase II in 100 ml of 1× PBS) at 37 °C for 60 min under slow rotation. Cells were collected and filtered with a cell strainer. The LPMCs were isolated by gradient density centrifugation in Percoll solution.

Generation of IL-10-producing CD4+ T Cells—The peripheral blood samples were obtained from six healthy volunteers (40 ml/subject) after obtaining informed consent. The mononuclear cells were isolated by gradient density centrifugation in Ficoll-Hypaque gradient separation medium. CD4+ CD25− T cells were further isolated with commercial reagent kits following the manufacturer’s instructions. The purity of CD4+ CD25− T cells was about 98% as determined by flow cytometry. The cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mm L-glutamine in a humidified atmosphere of 5% CO2 at 37 °C. The purified CD4+ CD25− T cells (1.5 × 10⁶/ml) were plated on culture plates and stimulated with immobilized anti-CD3 monoclonal antibodies (mAb) (1 µg/ml), anti-CD28 mAb (2 µg/ml), and recombinant IL-2 (50 units/ml) for 12 days. For IL-10-producing cell-inducing conditions, recombinant IL-4 (12.5 ng/ml) and anti-IFN-γ (5 µg/ml) were added. The cells were harvested and restimulated with anti-CD3 (5 µg/ml) and anti-CD28 (2 µg/ml) mAbs for 12 h.

Chromatin Accessibility by Real-time PCR—Referring to published procedures (21), 2 × 10⁶ nuclei in 100 µl of nuclear digestion buffer (10 mm Tris-HCl, pH 7.4, 15 mM NaCl, 60 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM CaCl2) plus 5 units/ml micrococcal nuclease were incubated at 25 °C for 10 min. 20 µl of stop solution (100 mM EDTA, 10 mM EGTA, pH 8.1) and 10 µl of 10% (w/v) SDS were added to terminate the reaction. DNA was purified by ethanol precipitation. Untreated micrococcal nuclease samples were used in PCR assays to measure the relative abundance of target regions by using the primer sets of the IL-10 promoter as shown above. To calculate the C1 value of each primer set, a standard curve was generated by using serial dilutions of genomic DNA. Chromatin accessibility values were calculated as a ratio of the undigested sample to the digested samples, and then the data were plotted as the ratio of accessibility observed in the unstimulated digested DNA samples.

Statistics—All values were expressed as the means ± S.D. of at least three independent experiments. The values were analyzed using the two-tailed unpaired Student’s t test when data consisted of two groups or by analysis of variance when three or more groups were compared. The correlation between variables was analyzed using Pearson’s correlation coefficient. p < 0.05 was accepted as statistically significant.

Reagents—The reagent list and some experimental procedures were presented in the supplemental material.

RESULTS

Levels of IL-10 Are Negatively Correlated with IL-23 in IBD Colon Mucosa—Immune deregulation in the intestine is one of the pathological features of IBD. IL-10 is one of the important cytokines in immune regulation (22). With the collected IBD colon mucosal specimens, we measured the levels of IL-10 in the extracted RNA and proteins. The results showed that the levels of IL-10 and the number of IL-10+ T cells were much less in the IBD group than in the control group (Fig. 1, A–C). In addition, among the IL-10+ T cells in Fig. 1C, we also observed that the frequency of CD4+ CD25+ Foxp3+ T regulatory cells was significantly lower in the IBD colon mucosa (1.22% ±
1.03%) than in the control group (10.2% ± 2.54%; p < 0.01, as compared with the IBD group).

Because IL-23 is a critical cytokine in the pathogenesis of IBD (5, 6), we also measured the expression of IL-23 in the collected colon specimens. The results showed that the levels of IL-23 and IL-23+ cells were markedly higher in the IBD group than in the control group (Fig. 1, A, B, and D). To clarify whether there was an association between the levels of IL-10 and IL-23 in the IBD colon mucosa, we performed a correlation assay with the ELISA data. The results showed a negative correlation between the levels of IL-10 and IL-23 in the IBD colon mucosa (r = −0.668, p < 0.05). The data suggest a potential that the decrease in IL-10 in the IBD colon mucosa may be induced by the increase in IL-23.

Frequency of Inflammatory Cells and Levels of MPO in IBD Colon Mucosa Are Negatively Correlated with Protein Levels of IL-10 and IgA—Using the same colon specimens as in Fig. 1, paraffin sections were prepared and stained with hematoxylin and eosin. The number of inflammatory cells (including PMN and mononuclear cells) in the sections was counted under a light microscope. The results showed that the frequency of inflammatory cells was significantly higher in the specimens from UC and CD than in normal colon tissues (Fig. 2A). The levels of MPO were markedly higher in IBD specimens than in the normal colon specimens (Fig. 2B). The correlation assay showed that the frequencies of PMN and mononuclear cells (Fig. 2A) in the IBD colon tissue were negatively correlated with the levels of IL-10 (Fig. 1) and IgA (Fig. 1) in the IBD colon mucosa (supplemental Table S2).

**FIGURE 1.** Expression of IL-10, IL-23, and IgA in IBD colon mucosa. Surgically removed colon tissues were obtained from IBD patients (nine UC patients; seven CD patients) and nine colon cancer patients (controls (Con)); the marginal normal tissue (proved by pathologists) was used in the experiments. The samples were analyzed by ELISA, quantitative RT-PCR, Western blotting, and flow cytometry individually. A, B, and E, the bar graphs indicate the levels of mRNA (A) and proteins (B) of IL-10 and IL-23 and the levels of total IgA (E). Data are presented as means ± S.D. *, p < 0.05, as compared with the control group. In C1, D1, and F1, the dot plots show CD4+ T cells (C1), CD4+ (C1), macrophages (D1), and plasma cells (F1), respectively; the gated cells in the panels were further analyzed for the expression of IL-10 (C2–C4), IL-23 (E2–E4), and IgA (F2–F4). The tissue types are annotated above histograms. Isotype controls are shown in C5, D5, and F5. Samples obtained from patients were analyzed individually.

**FIGURE 2.** Frequencies of inflammatory cells and MPO are increased in IBD colon mucosa. All samples used in Fig. 1 were also analyzed by H&E staining. A, the bars indicate the frequencies of PMN and mononuclear cells that were counted under a light microscope (counted in 20 fields per sample at ×400) in colon tissue. B, the bars indicate the levels of MPO in colon tissue extracts. The data were presented as means ± S.D. *, p < 0.01, as compared with controls (Con).
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In vitro production of IL-10 and IgA by isolated LPMCs. LPMCs were isolated from the samples in Fig. 1. The cells were cultured in the present or absence of phorbol 12-myristate 13-acetate (PMA; 50 ng/ml) (Medium group) and ionomycin (1 μg/ml) with or without anti-IL-23 antibody (αIL-23; 100 ng/ml) for 3 days. A and B, the bars indicate the levels of IL-10 (A) or IgA (B) in the supernatants as determined by ELISA. Specimens from different patients were assessed individually. *, p < 0.01, as compared with the control group (Con). #, p < 0.01, as compared with the medium group. Inactive, cells were not activated by phorbol 12-myristate 13-acetate/ionomycin. Isotype, cells were treated with isotype IgG instead of αIL-23. αIL-23/αIL-10, Cells were treated with antibodies of both IL-23 and IL-10.

IBD colon specimens (Fig. 1), LPMCs were isolated from the colon specimens (using the same batch of specimens as in Fig. 1) and were cultured in the presence of phorbol 12-myristate 13-acetate and ionomycin for 3 days. The cell viability was more than 90% as checked by the trypsin blue exclusion assay before collecting the samples. The supernatants were analyzed by ELISA. The results showed that the levels of IL-10 in the supernatant were significantly less in the IBD group than in the control group (Fig. 3A). Considering that IL-23 (Fig. 1) might suppress the expression of IL-10 in CD4+ T cells, we added the antibodies of IL-23 to the culture to neutralize the IL-23 in separate experiments. Indeed, the addition of IL-23 antibody markedly increased the levels of IL-10 in the supernatants (Fig. 3A). The results imply that the existence of IL-23 plays an important role in the suppression of IL-10 in IBD mucosa. We also observed that the levels of IgA were lower in IBD samples than in controls that were elevated after the addition of anti-IL-23 antibody to the culture, which was abolished by the concurrent addition of anti-IL-10 antibody (Fig. 3B). These data suggest that IL-23 inhibits IL-10 expression; the latter in turn inhibits IgA production. This inference is supported by the published data that IL-10 has the capacity to induce IgA production via activating the signal transducer and activator of transcription 3 (STAT3) pathway (23).

Gene Transcription of IL10 Was Suppressed in CD4+ T Cells of IBD Colon Mucosa—We next observed the IL10 gene transcriptional status in CD4+ T cells of the colon specimens. The results of the ChIP assay showed that markedly less acetylated histone H3 and H4 was detected in the samples from both UC and CD as compared with normal controls (Fig. 4A). The promoter accessibility of IL-10 was much weaker (Fig. 4B), and the levels of IL-10 mRNA (Fig. 4C) and IL-10 protein (Fig. 4D) were significantly lower in IBD specimens than in controls. When the activated CD4+ T cells were treated with anti-IL-23 anti-body, the IL10 gene transcriptional status was significantly up-regulated.

IL-23 Suppresses Expression of IL-10 in Th2 Cells—To further understand the role of IL-23 in the suppression of IL-10 in the IBD intestinal mucosa, we generated polarized Th2 cells; the Th2 cells were exposed to IL-23 at graded doses in culture for 3 days. The cells were collected at the end of culture and analyzed by ChIP assay. The results showed that IL-23 significantly suppressed the levels of acetylated histone H3/H4 (Fig. 5A), IL10 gene transcription is compromised in IBD mucosa. Published data also demonstrate that much less IgA was detected in the IBD intestinal mucosa than in normal controls. This fact implies that the IBD intestinal mucosa produces much less IgA as compared with normal intestinal mucosa.

Upon exposure to microbes or microbial products, the plasma cells synthesize IgA (15, 24). One of the major functions of IgA is to keep bacteria from directly contacting the epithelial cells. IgA is an important component in the defensive barrier in the intestinal mucosa (15). Without the presence of IgA, the bacteria have more robust capacity to colonize on the local tissue to evoke inflammatory responses (25). Our data show that much less IgA was detected in IBD colon tissue. This fact implies that the so-called “defensive barrier of IgA” has been compromised in the IBD colon mucosa. The subsequent data showed that the frequencies of PMN and mononuclear cells and MPO levels are increased in the IBD colon mucosa, which also indicates that the defensive system of the colon mucosa in IBD patients is weakened. Published data also demonstrate that

FIGURE 3. In vitro production of IL-10 and IgA by isolated LPMCs. LPMCs were isolated from the samples in Fig. 1. The cells were cultured in the presence or absence of phorbol 12-myristate 13-acetate (PMA; 50 ng/ml) (Medium group) and ionomycin (1 μg/ml) with or without anti-IL-23 antibody (αIL-23; 100 ng/ml) for 3 days. A and B, the bars indicate the levels of IL-10 (A) or IgA (B) in the supernatants as determined by ELISA. Specimens from different patients were assessed individually. *, p < 0.01, as compared with the control group (Con). #, p < 0.01, as compared with the medium group. Inactive, cells were not activated by phorbol 12-myristate 13-acetate/ionomycin. Isotype, cells were treated with isotype IgG instead of αIL-23. αIL-23/αIL-10, Cells were treated with antibodies of both IL-23 and IL-10.

FIGURE 4. IL-10 gene transcription is compromised in IBD mucosa. CD4+ T cells were isolated from the samples in Fig. 1. Cells were analyzed by a ChIP assay. A, the gel graphs show acetylated histone H3 and H4. B, the bars indicate the mRNA levels of IL-10 in activated CD4+ T cells. C, the bars indicate the levels of activated promoters of IL-10. D, micrococcal nuclease accessibility by real-time PCR was performed on the nuclei of the CD4+ T cells. The y axis indicates the amount of PCR products, and the x axis indicates the sources of the samples. *, p < 0.01, as compared with the control group (Con).
mice with high levels of IgA in the intestine show enhanced resistance to microbial products (26).

IL-10-deficient mice develop spontaneous enterocolitis (27, 28). The underlying mechanism by which defective IL-10 signaling leads to intestinal inflammation is unknown. Published data indicate that IL-10 is an important cytokine in the induction of IgA, mainly by the mechanism of expanding the IL-21-induced IgA production in plasma cells (29). IL-10 also plays a role in facilitating the differentiation of plasma cells (30). Administration of IL-10-secreting probiotics increases the IgA levels and prevents food allergen-related inflammation in the intestine (31). Peyer’s patch CD3+ cells are primarily involved by favoring IgA production through the release of IL-10 and TGF-β (32). Our data are in line with these studies by showing that the levels of IL-10 are decreased in the IBD colon mucosa. These data further demonstrate that the decrease in IL-10 is positively correlated with the decrease in IgA. IgA is the most important antibody in preventing bacterial infection in the intestine (15). Our data, at least partially, elucidate the mechanism by which the deficiency of IL-10 contributes to intestinal inflammation as reported previously (27, 28).

In this study, we measured the levels of IL-10 in colon biopsy-derived CD4+ T cells. CD4+ T cells include Th1, Th2, regulatory T cell (Treg), and Th17 cells. IL-10 can be produced by Th2 cells or Tregs. In other words, the sources of IL-10 in the microenvironment of the colon mucosa can be either Th2 cells or Tregs or other cell types (such as tolerogenic dendritic cells). IL-10 from different cell types in the intestine is supposed to have a similar effect on the production of IgA. Because we observed less IL-10 expression in IBD specimens, we wondered whether the decrease in IL-10 also affected the production of IgA in the existing plasma cell in the IBD colon mucosa. These data support our postulation by showing not only much lower frequency of IgA+ plasma cells in the IBD colon mucosa but also showing that lower levels of IgA were produced by the existing plasma cells as compared with non-IBD controls. It is known that IL-10 plays a role in the production of IgA mainly via promoting the differentiation of plasma cells (29). Based on the fact that within the 3-day culture, the production of IgA is increased, this either means that new plasma cells are being differentiated during the culture period or that IL-10 is having a previously unappreciated role; for example, IL-10 may also facilitate the production of IgA by existing plasma cells. The underlying mechanism is to be further investigated.

The increase of IL-23 in IBD has been well documented (5, 6). A subset of lymphocyte overexpression of the IL-23 receptor has been noted in the IBD intestinal mucosa. These lymphocytes over-respond to the stimulation of IL-23 and produce abundant proinflammatory cytokines; this may be one of the causes of susceptibility to IBD (5, 33). Our data are consistent with these studies by showing high levels of IL-23 in the IBD colon mucosa. The data also provide further information that the increase in IL-23 is negatively correlated with the decrease in IL-10 and IgA in the IBD colon mucosa; these findings imply that IL-23 may suppress the expression of IL-10 in the intestine of IBD patients. Indeed, this postulation is supported by further experimental results. In the in vitro experiment, we observed that activated LPMCs from IBD patients had markedly lower secretion of IL-10 than controls; blocking IL-23 dramatically increased the levels of IL-10 in the culture. This result is supported by published experiments; Correal et al. (34) showed that in a multiple sclerosis animal model study, inhibition of IL-23 significantly increased the expression of IL-10. The present data also reveal that histone H3 and H4 acetylation in the IL10 gene region, IL10 promoter accessibility, IL10 promoter activity, and IL-10 mRNA expression are significantly inhibited in the CD4+ T cells isolated from the IBD colon mucosa. This phenomenon can be also replicated by a cell culture study, as shown by the present data.

Regulatory T cells (Tregs) are critical mediators of peripheral immune tolerance; they contribute to immune homeostasis in the intestine. Functional deficiency of Treg is involved in the pathogenesis of a number of immune disorders (35). Diverse data about Tregs in IBD have been reported. Wang et al. (36) recently indicated that the frequency of Tregs was increased in the peripheral blood and the IBD intestinal mucosa. This phenomenon implies that Treg function is compromised in IBD patients. Boschetti et al. (37) revealed that the frequency of CD4+ CD25+ Foxp3+ Treg was significantly lower in active IBD patients than in controls. Blocking IL-23 dramatically increased the levels of IL-10 and IgA in the IBD colon mucosa; these findings imply that IL-23 may suppress the expression of IL-10 in the intestine of IBD patients. Indeed, this postulation is supported by further experimental results. In the in vitro experiment, we observed that activated LPMCs from IBD patients had markedly lower secretion of IL-10 than controls; blocking IL-23 dramatically increased the levels of IL-10 in the culture. This result is supported by published experiments; Correal et al. (34) showed that in a multiple sclerosis animal model study, inhibition of IL-23 significantly increased the expression of IL-10. The present data also reveal that histone H3 and H4 acetylation in the IL10 gene region, IL10 promoter accessibility, IL10 promoter activity, and IL-10 mRNA expression are significantly inhibited in the CD4+ T cells isolated from the IBD colon mucosa. This phenomenon can be also replicated by a cell culture study, as shown by the present data.

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logical state of mucosal lymphocytes in the bulk of IBD patients, who typically have more quiescent disease.

Collectively, the present data show that the levels of IL-10 and IgA and the frequency of CD4+ CD25+ Foxp3+ IL-10+ Tregs are significantly lower in the IBD colon mucosa. The IL-23 levels are markedly higher in the IBD colon mucosa, which is positively correlated with the inflammatory components (PMN, mononuclear cells, and MPO levels). IL-23 can inhibit the gene transcription of IL-10 in the immune cells isolated from the IBD colon mucosa. We conclude that the over-expression of IL-23 in the intestinal mucosa weakens the defensive barrier (the IgA) in the gut and disturbs the immune regulatory machinery (IL-10 and Tregs).

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