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Original Article

IgG4+ plasma cell infiltration is correlated with the development of inflammatory bowel disease and can be regulated by TLR-4

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Abstract: Immunoglobulin 4 (IgG4) is commonly considered a hallmark of autoimmune pancreatitis (AIP). Inflammatory bowel disease (IBD) is believed to play a substantial role in the setting of AIP. Toll-like receptor 4 (TLR4) plays an important role in inflammation. The relationship between IgG4 and TLR4 in the process of IBD is incompletely explored. Our study aimed to assess the expression of IgG4 and TLR4 in IBD patients and to find the role of IgG4 and TLR4 in the IBD process. A cohort of 68 IBD patients was enrolled in our study, and 20 healthy persons served as a control group. Intestinal IgG4 positive (IgG4+) plasma cell infiltration was measured by immunohistochemistry. Serum IgG4 and TLR4 levels were measured by ELISA. Fifteen additional features from the patients' general medical information and lab data were also collected to assess the risk factors of IBD activity by logistical analysis. BALB/c mice were used to build a rat IBD model with dextran sulfate sodium (DSS). The TLR4 inhibitor TAK242 was used to regulate the expression of TLR4. The expression of IgG4 and TLR4 in serum was detected by ELISA. The expression of IgG4 and TLR4 in the intestines were assayed with western blot. Our results revealed that the infiltration of IgG4+ plasma cells was higher in IBD patients (14/68 vs 0/20, P<0.05). The incidence of IgG4+ plasma cells in the IBD group (48.5%) was higher than in the control group (33/68 vs 0/20, P<0.05). Serum IgG4 and TLR4 levels in the IBD group were significantly higher compared with the control group (P<0.05). Based on our logistical analysis, three variables: IgG4+ plasma cell infiltration, CRP, and HB were identified as independent risk factors with odds ratios of 10.917, 1.031, and 0.923, respectively (*P*<0.05). After the TLR4 was suppressed, the infiltration of IgG4+ plasma cells in the intestines decreased significantly, and expression of IgG4 in the serum and intestines was suppressed. This study demonstrated that intestinal IgG4+ plasma cell infiltration was higher in IBD patients than in the control group. IgG4+ cell infiltration is significantly enhanced in ulcerative colitis patients. IgG4+ plasma cell infiltration can be regulated by TLR4, and an increase of IgG4+ plasma cell infiltration, CRP, and anemia are correlated with an increased risk of active IBD.

Keywords: Immunoglobulin G4, toll-like receptor 4, inflammatory bowel disease, Crohn's disease, ulcerative colitis, activity

Introduction

Immunoglobulin G4 (IgG4) was recently linked to autoimmune pancreatitis (AIP). Inflammatory bowel disease (IBD) is a group of chronic gastrointestinal disorders characterized by intestinal inflammation, including Crohn's disease (CD) and ulcerative colitis (UC). The prevalence of AIP in IBD patients is much higher than in the general population (6% versus 0.4%-0.5%). Ravi et al. demonstrated that patients with both AIP and IBD may have an increased extent and severity of IBD. The existence of IBD is associated with the finding of IgG4-positive cells in colon biopsies and may represent an extra-pancreatic manifestation of AIP [1]. However, others reported a significant infiltration of IgG4 cells in colonic pinch biopsies in a subset of patients with IBD without coexistent AIP (24%) [2, 3]. IgG4+ plasma cell infiltration has been observed in the colonic mucosa of patients with ulcerative colitis. The expression of IgG4 in IBD patients remains to be elaborated. A study demonstrated that infiltration of IgG4+ plasma cells appeared to be associated with chronic pouch inflammation and concur-

rent autoimmune diseases [4]. Infiltration of IgG4+ plasma cells was detectable in the colonic mucosa of UC patients and was reported to be associated with disease activity [5].

Toll-like receptors (TLRs) are evolutionarily conserved transmembrane receptors usually expressed by antigen presenting cells. TLRs play an important role in the digestive system, for they can recognize invading microbes in the intestinal barrier and activate the immune response [6-8]. Sustained hyper-activation of TLRs may lead to chronic inflammation in IBD [9]. Toll-like receptor 4 (TLR4) is a transmembrane protein, a member of the toll-like receptor family. TLR4 is an important protein involved in non-specific immunity and a bridge between non-specific immunity and specific immunity [10, 11]. Research indicates that TLR4 signaling mediates IL-17 expression and plays an important role in chronic inflammatory diseases [12].

However, the mechanism for IgG4 and TLR4 regulating IBD development is undetermined. In our study, we hypothesized that IgG4 expression may exacerbate disease activity in IBD patients. A cohort of 68 IBD patients was enrolled, and 20 healthy persons served as a control group. Serum IgG4 level and intestinal IgG4+ cells were quantitatively assessed in both groups. 15 additional features of the patients' general medical information and lab data were also collected to assess potential risk factors of IBD activity using logistical analysis. The TLR4 inhibitor TAK242 was used to regulate the expression of TLR4 in rats, and the relationship between IgG4 and TLR4 in the process of IBD was studied.

Materials and methods

Experimental subjects

From January, 2013 to December, 2016, 68 IBD patients were enrolled in our study, including 40 cases of CD (Crohn's Disease) and 28 cases of UC (uylcerative colitis). Another 20 healthy cases were selected as a control group at the same time. Inclusion criteria were: age >18 years old; clinically diagnosed with IBD and confirmed by imaging and pathology; absence of ischemic bowel disease or non-steroidal anti-inflammatory drugs (NSAIDs) related bowel disease; no history of infectious diseases in the previous 3 months; no history of intestinal tuberculosis; no history of malignant tumors; no history of severe cardiopulmonary disease. Informed consent was obtained for all subjects. The study was approved by the Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University.

Comparative assessment analysis

Demographic data, clinical information, and laboratory data were recorded, including gender, age, disease duration, bowel frequency, abdominal pain, involved lesion sites, body mass index (BMI), hemoglobin (HB), erythrocyte sedimentation rate (ESR), C-reaction protein (CRP), IgG4+ plasma cell infiltration, extra-intestinal manifestation (EIM), serum IgG4, presence of postcholecystectomy syndrome (PCS), and AIP.

'Disease duration' is defined as the course of disease onset to the first treatment time, counted by month. 'Bowel frequency' is defined as the mean number of defecations per day in a week. 'Involved lesion sites': means that in CD, the rectum, the colon, and the small intestine are considered three separated parts. In UC, the rectum, the left colon, and the right colon are considered three separate parts. 'Extra-intestinal manifestation (EIM)' includes arthritis, vasculitis, erythema nodosum, etc. [13].

Disease activities were measured using the Crohn's disease activity index (CDAI) for CD [7] and the Mayo Score for UC [14]. The CDAI assessed the parameter of stool frequency, abdominal pain, EIM, fistula, abdominal mass, use of anti-diarrheal drugs, anemia and weight loss. The Mayo scores measured diarrhea, rectal bleeding, findings on endoscopy, and the physician's global assessment of disease severity. Higher scores represent greater disease severity. Next, all 68 IBD patients were divided into an active group (defined as a CDAI change of >150 points or a Mayo score of >2 in UC, n=39) and a non-active group (n=29).

Animal experiment

A total of 30 female BALB/c nude mice (6-week-old, weighing 20±2 g) were purchased from the Center of Experimental Animals of Wenzhou Medical University (Wenzhou, China). TAK242 was purchased from Merck Millipore (Merck

Table 1. General information of IBD patients and control group

	IBD group	Control group	*P* value
Gender			0.371
Male	48	12	-
Female	20	8	-
Age (years)	35.21±14.12	38.8±4.3	0.428
Fistula	24	0	-
Extra-intestinal manifestation	3	0	-
Arthritis	1	0	
Vasculitis	1	0	
Erythema nodosum	2	0	

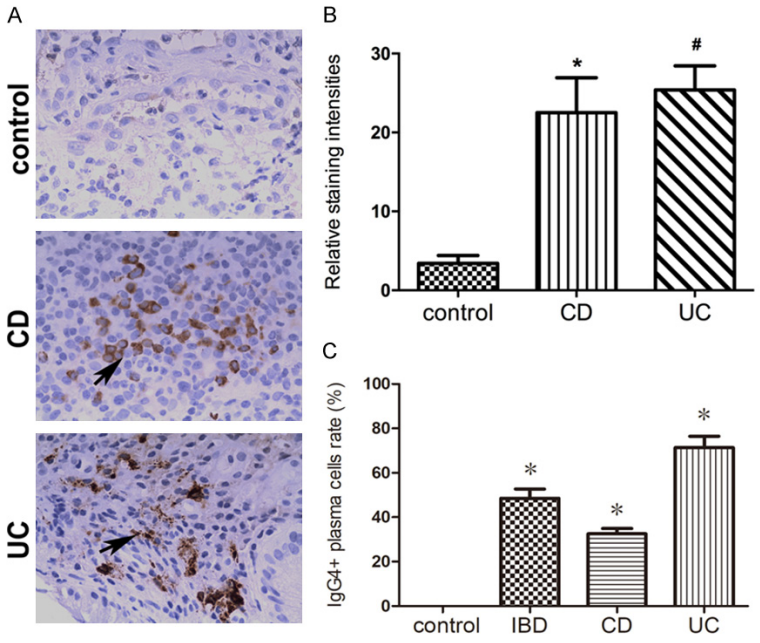

Figure 1. Intestinal IgG4+ plasma cell infiltration by immunohistochemistry in ulcerative colitis patients. (HP magnification ×400 [0.2 mm^2]). A, B. Plasma cells in the lamina propria were considered IgG4+ in the presence of brown staining. Areas with the highest density of IgG4+ plasma cells were identified at high-power field (HPF). C. Infiltration of IgG4+ plasma cell in the control and IBD groups. Infiltration of IgG4+ plasma cells is higher in IBD patients (33/68 vs 0/20, $^{*}P<0.05$). IgG4+ plasma cells in CD group (13/40 vs 0/20, $^{*}P<0.05$) and UC group (20/28 vs 0/20, $^{**}P<0.05$) were significantly higher compared with the control group. $^{*}P<0.05$, by Pearson Chi-Square test.

Millipore, Germany). The mice were housed under a 12 h light/dark cycle with a constant temperature of 22±1°C and a humidity of 55%. No dietary restriction was applied. All the animal experiments were approved by the Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University, and all efforts were made to minimize suffering. Experimental colitis was induced by giving mice drinking water containing 5% (w/v) dextran sulfate sodium (DSS, Sigma, USA) for 7 days. Mice were randomly divided into 3 groups (n=10/group), including a blank control group, a DSS group, and a DSS + TAK242 group (3 mg/kg). TAK242 were administered once a day by intraperitoneal injection 3 days before DSS treatment, and the blank control group and the DSS group were administered with an equal volume of phosphate buffered saline (PBS). At day 7 following induction with DSS, blood samples were collected for an ELISA assay, and the mice were killed by CO_2 inhalation, and then the entire colons were collected and stored at -80°C until analysis.

ELISA assay

Blood samples were obtained and serum IgG4 levels were measured using the ELISA method in our hospital chemistry laboratory. An elevated concentration of serum IgG4 was defined as a value above 2.01 g/L, according to the standard reference used in First Affiliated Hospital of Wenzhou Medical University (ranges from 0.03 g/L to 2.01 g/L).

Immunohistochemistry

Intestinal tissue samples were collected by colonoscopy and prepared as 4 μm, paraffin-embedded tissue sections. The expression of IgG4 was measured by immunohistochemistry (IHC) (Rabbit-anti-human monoclonal antibodies, 1:300 dilutions, Invitrogen, USA) according to the instruction manual. The stained slides were evaluated independently by two experienced pathologists. Plasma cells in the lamina propria were considered IgG4+ in the presence of discrete cytoplasmic

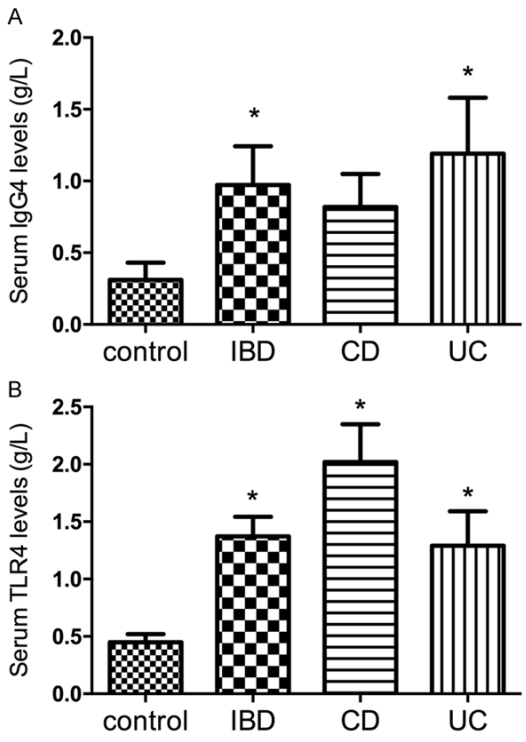

Figure 2. Expression of IgG4 and TLR-4 in serum. Compared with the control group, $^{*}P<0.05$.

brown staining. Areas with the highest density of IgG4 positive plasma cells were used for analysis. For each sample, 2-3 slides were evaluated, and an average number of IgG4 positive cells per high-power field (HPF) was determined. Based on other studies, a count of less than 10 was considered negative [15].

Western blotting

Western blotting experiments were carried out using a previously standardized protocol. Intestinal tissues were collected by lysis buffer to extract the total protein of cells. After the protein was purified and quantified, 50 μg of estimated protein per sample was loaded on to 10% SDS-PAGE gels. After PVDF membrane transfer, blocking was done with 2% BSA, and then TLR-4 (1:500 dilutions, Abcam, USA), IgG4 (1:500 dilutions, Abcam, USA), IL-6 (1:500 dilutions, Abcam, USA) and NF-kB (1:500 dilutions, Abcam, USA), and then the primary antibodies were incubated overnight at 4°C. HRP-labeled secondary antibodies were incubated at room temperature. Blots were developed using the chemiluminescent substrate (Millipore, USA). The expression levels of TLR-4, IgG4, IL-6, and NF-kB were standardized by GAPDH.

Statistical analysis

Descriptive statistics were calculated for all variables. These included the mean and standard deviation for continuous variables and frequency and percentage for categorical variables. Comparison of continuous variables was valued by Wilcoxon's tests; the association of categorical variables was valued by Pearson's Chi-square or Fisher's exact tests. A *P*-value less than 0.05 was defined as significant. According to the evaluation of disease activity by CDAI and Mayo scores, IBD patients were divided into an active group (defined as a CDAI change of >150 points or Mayo score of >2 in UC, n=39) and an inactive group (n=29). Binary logistic regression was used to determine independent predictive factors of IBD disease activity between the two groups. Statistical analyses were performed using SPSS version 13.0.

Results

General information

From January, 2013 to December, 2016, sixty-eight IBD patients were involved in our study. Among them, 40 cases were CD (Crohn's disease) and 28 cases were UC (ulcerative colitis). 48 were male and 20 were female. The mean age was 35.21±14.12 years old. In the control group, 12 were male and 8 were female. The mean age was 38.8±4.3 years old. Twenty-four patients also suffered from a fistula. Four patients had an extra-intestinal manifestation (EIM) including one with arthritis, one with vasculitis, and two with erythema nodosum (**Table 1**).

Infiltration of intestinal IgG4+ plasma cells increased in ulcerative colitis patients

In patients with IHC, plasma cells were considered IgG4 positive in the presence of cytoplasmic brown staining (**Figure 1A**, **1B**). Among the 68 IBD patients (40 CD patients and 28 UC patients), a total of 33 samples were positive for IgG4+ plasma cell infiltration, of which 13 were in the CD group (32.5%) and 20 were in the UC group (71.4%). In the control group, 20 cases of intestinal samples provided IgG4 negative results. The incidence of IgG4+ plasma cells in the IBD group (48.5%) was higher than in the control group (33/68 versus 0/20, P<0.05) (**Figure 1C**).

Table 2. Comparison of factors in active group and inactive group

Variables	Group		*P*-value
	Inactive (n=29)	Active (n=39)	
Male Gender	22 (75.8%)	26 (66.7%)	0.776
Age, years	35.31±14.7	35.13±13.0	0.958
Disease duration, months	21.4±30.5	20.5±30.0	0.905
Bowel frequency, times	3.1±2.8	4.2±2.5	0.106
Abdominal pain, times	1.2±0.8	1.5±0.9	0.404
BMI	20.2±2.87	19.4±2.61	0.261
Hemoglobin (g/L)	122±12.7	107±19.2	0.001*
Erythrocyte sedimentation rate (mm/H)	17.9±9.39	28.8±19.21	0.003*
CRP (g/L)	17.61±23.7	35.63±31.8	0.013*
IgG4+ plasma cell infiltration (%)	1 (3.4%)	13 (33.3%)	0.014*
Involved lesion site	1.7±0.70	2.1±0.78	0.080
EIM	0	4 (10.3%)	0.075
PCS	0	0	NS
AIP	0	0	NS

EIM, extra-intestinal manifestation; PCS, postcholecystectomy syndrome; AIP, autoimmune pancreatitis; NS, no significance. *P<0.05.

Table 3. Risk factors of IBD activity on binary logistic regression

Variable	B value	Odds ratio	95% Confidence interval	*P* value
IgG4+ infiltration	2.390	10.917	[1.053, 113.210]	0.045*
CRP	0.030	1.031	[1.0, 1.062]	0.047*
HB	-0.08	0.923	[0.873, 0.977]	0.005*
ESR	0.032	1.032	[0.988, 1.078]	0.155

*: P<0.05.

Serum IgG4 and TLR-4 levels increased in IBD patients

Serum IgG4 and TLR-4 levels were detected in the IBD group and the control group. The results indicated that the serum average IgG4 and TLR-4 values in the IBD group was higher than in the control group (P<0.05) (**Figure 2**). These results indicated that IgG4 and TLR-4 had strong positive correlation.

Comparative assessment of variables for IBD activity

A set of 15 variables related to the patients' demographic and clinical information and lab data were recorded, including gender, age, disease duration, bowel frequency, abdominal pain, number of involved lesion sites, BMI, HB, ESR, CRP, extra-intestinal manifestation (EIM), and the presence of PCS and AIP. Data from the non-active group and rhw active group were analyzed by T-test or Chi-square test. Results are summarized in **Table 2**. Four variables, including IgG4+ plasma cell, ESR, CRP, and hemoglobin, demonstrated a significant statistical difference (P< 0.05).

Binary logistic regression analyses of variables for IBD activity

The activity state of IBD was considered the dependent variable, and the statistically significant variables were set as covariates. Next, the data was analyzed by binary logistic regression. Four variables were accessed in the equation, with three variables retained as independent risk factors (**Table 3**). The odds ratio (OR) value of IgG4+ infiltration, CRP, and hemoglobin were 10.917, 1.031, and 0.923, respectively (P<0.05). This reveals that an increase of IgG4+ infiltration incidence and CRP levels are correlated with an increased risk of active IBD, while a decrease of hemoglobin concentration enhances the risk of active IBD.

TAK242 can suppress the infiltration of IgG4+ plasma cells, and inhibit the expression of IgG4 in intestinal

BALb/c mice were used to build the ulcerative colitis model, and the results of immunohistochemistry showed the level of IgG4+ positive cells in the IBD group was higher than in the control group, but the treatment of TAK242 can reverse this trend (P<0.05) (**Figure 3A**, **3B**). In addition, ELISA results indicated TAK242 can inhibit the expression of IgG4 in serum (**Figure 3C**). These results suggest that the expression of IgG4 can be regulated by TLR4 in the intestine and serum.

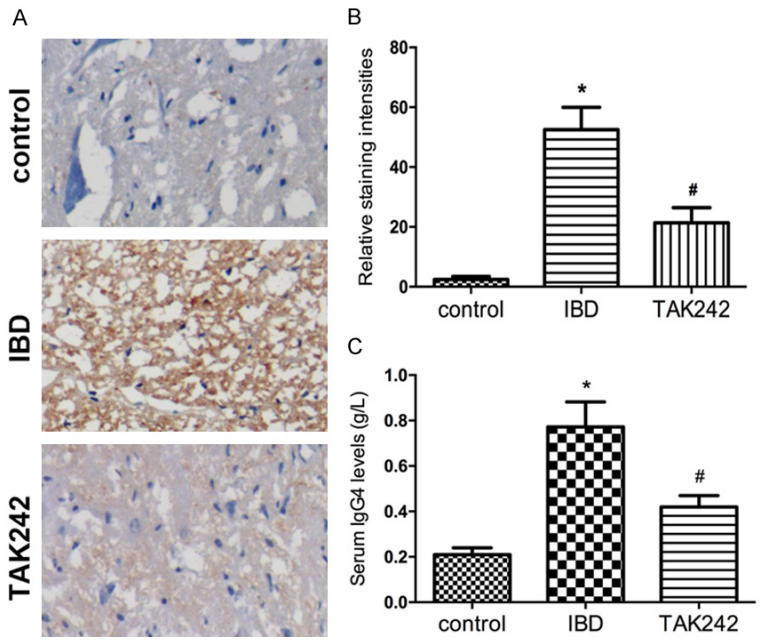

Figure 3. The effect of TAK242 on IgG4 expression in BALb/c mice intestinal. A, B. Immunohistochemistry was used to detect the expression of IgG4. Plasma cells in the lamina propria were considered IgG4+ in the presence of brown staining. Areas with the highest density of IgG4+ plasma cells were identified at high-power field (HPF). C. Serum IgG4 levels were assayed with ELISA. Compared with the control group, $^{*}P<0.05$; Compared with IBD group, $^{\#}P<0.05$.

TLR4 inhibitor TAK242 can inhibit the expression of IgG4 in the intestines

In order to explore the interaction between IgG4 and TLR4 in IBD patients and find the mechanisms of IgG4 and TLR4 in the IBD process, western blot was used to detect the expression of TLR-4, IgG4, IL-6, and NF-kB in mice intestines. The results showed the expression TLR-4, IgG4, IL-6, and NF-kB in the intestines can be downregulated by TAK242 (**Figure 4**). These results indicate that IgG4+ plasma cell infiltration can be regulated by TLR-4, and TLR-4 can be a key factor in the process of IBD.

Discussion

IgG4 is known as a blocking antibody in IgE mediated allergies and parasite infections [16]. Recently, a few studies demonstrated a significant increase of serum IgG4 levels and IgG4+ plasma cell infiltration in autoimmune pancreatitis patients [17]. The infiltration of IgG4+ plasma cells were detected more frequently in the extrapancreatic organs (such as the lungs, kidney, etc.), making IgG4-related diseases, characterized by tissue fibrosis and remodeling [18]. Others reported a low incidence of IgG4 infiltration in the gastrointestinal tract [19]. In our study, we assessed the expression of IgG4 in IBD patients. The IgG4 serological expression coincides with the histological expression. Compared with the control population, the serum IgG4 level of the IBD group was significantly increased (P<0.05). And intestinal IgG4+ plasma cells were higher in IBD patients (P< 0.05). The incidence of IgG4+ plasma cells was also enhanced in the UC group (P<0.05). Our results are similar to the findings of Takahiro [20].

Previous studies also suggest that IgG4 may play an important role in IBD activity. Navaneethan investigated the tissue infiltration of IgG4 plasma cells in symptomatic patients with ileal pouch-anal anastomosis, concluding that IgG4+ cells appeared to be associated with chronic inflammation and a concurrent disorder [21]. We divided IBD into non-active and active groups by the CDAI and Mayo scores. Based on 15 variables between the two groups, we revealed that IgG4+ plasma cell infiltration is significantly increased in the active group (P<0.05). Additionally, IgG4+ cell infiltration is an independent risk factor for disease activity (OR=10.917, P<0.05), indicating that the IBD patients with IgG4 infiltration are at higher risk of active IBD. Moreover, two other independent factors of active IBD were CRP and anemia. CRP is known as one of many acute phase proteins that increase in the serum of patients with acute phase IBD [22]. One of the early studies of IBD showed a close correlation between CRP and clinical activity [23]. Another review from Vermeire showed that both CRP and ESR correlated well with disease activity [24]. Our study illustrated a significant increase of CRP and ESR in the active group (P<0.05). However, only CRP appeared to be an independent risk factor based on logistical analysis (OR=1.031, P<0.05).

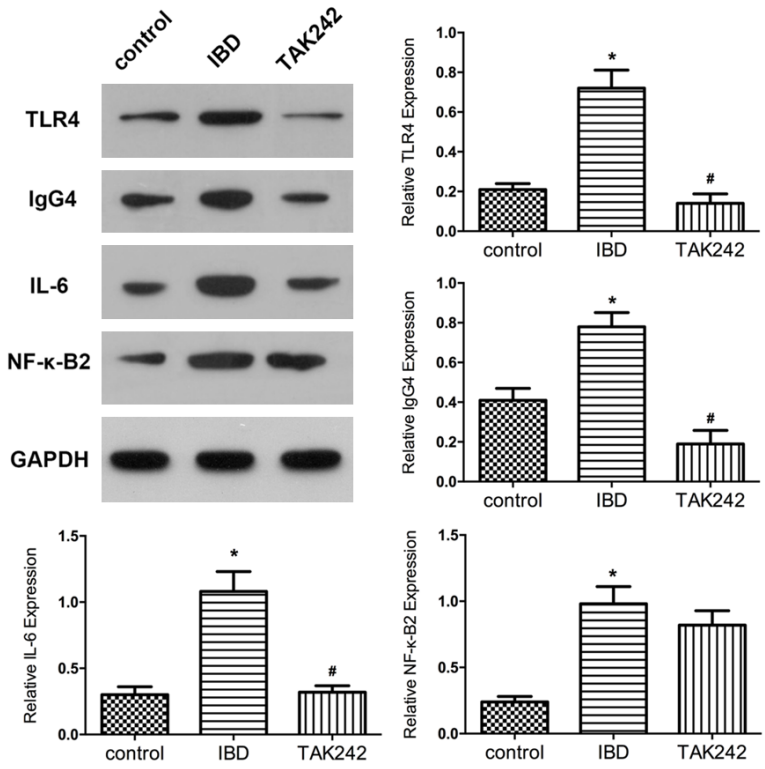

Figure 4. Expression of TLR-4, IgG4, IL-6 and NF-kB were detected with western blot in the intestines. Compared with the control group, $^{*}P<0.05$; Compared with IBD group, $^{\#}P<0.05$.

TLRs were shown previously to play a central role in mucosal innate immune regulation, and they are important immune receptors that participate in the recognition of pathogen-associated molecular patterns and the activation of signal transduction pathways of antimicrobial genes, by identifying and binding to small molecular components on pathogens [25]. TLR4 signaling in immune cells of the colonic mucosa plays a central role in maintaining a chronic inflammatory state by producing inflammatory cytokines [26]. In addition, some studies have shown that the expression of TLR4 is also associated with the occurrence and development of colitis-related tumors, and therefore closely related to the pathogenesis and progression of IBD [27]. TAK242 is one of the specific TLR4 inhibitors, and, in this study, TAK242 was used to regulate the expression of TLR4. After the expression of TLR-4 was inhibited, the expression of IgG4 in tissue and serum was detected. Our study results indicated the expression of IgG4, IL-6 and NF-kB can be downregulated by TAK242. These findings suggested TLR4 may participate in the IBD process to influence the expression of IgG4.

In conclusion, our research results indicate that intestinal IgG4+ plasma cell infiltration in IBD patients is higher than in the control group, and serum IgG4 level was also significantly enhanced in IBD patients. In addition, IgG4+ plasma cell infiltration can be regulated by TLR4, and IgG4+ plasma cell infiltration, CRP, and anemia were three independent risk factors of disease activity.

Acknowledgements

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Disclosure of conflict of interest

None.

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