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ORIGINAL ARTICLE—ALIMENTARY TRACT

Food antigen-induced immune responses in Crohn's disease patients and experimental colitis mice

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Abstract

Background In Crohn's disease (CD), the involvement of food antigens in immune responses remains unclear. The objective of this study was to detect immune responses against food antigens in CD patients and examine the mechanism in a mouse model of colitis.

Methods We enrolled 98 CD patients, 50 ulcerative colitis patients, and 52 healthy controls (HCs) to compare the levels of serum immunoglobulin (Ig)Gs against 88 foods. The presence of serum IgGs against foods was also examined in interleukin (IL)-10 knockout (KO) mice in which $CD4^+$ T cell activation by antigenic food protein was assessed. Mice transferred with IL-10 KO cells received diets with or without food antigens, and the development of colitis was evaluated.

Results The prevalence of IgGs against various foods, especially vegetables, grains, and nuts, was significantly higher in CD patients than in HCs. Similarly, the prevalence of IgGs against food proteins was higher in IL-10 KO mice than in BALB/c mice. Beta-conglycinin, identified as an antigenic food proteins in IL-10 KO mice, induced $CD4^+$ T cell production of interferon-γ and IL-17 through dendritic cell antigen presentation. Elimination of the food antigens ameliorated the development of colitis in mice without altering the composition of their intestinal microbiota.

Conclusions In CD colitis mice, intestinal inflammation via $CD4^+$ T cell hyperactivation was induced by food antigens associated with high serum IgG levels and was ameliorated by the elimination of food antigens. This disrupted immunological tolerance to food antigen, which might act as an exacerbating factor, remains to be elucidated in CD patients.

Keywords Crohn's disease · Food antigens · Serum immunoglobulin G · $CD4^+$ T cell · Experimental colitis

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Introduction

Inflammatory bowel diseases (IBDs) such as Crohn's disease (CD) and ulcerative colitis (UC) are chronic disorders of the intestinal mucosal immune system caused by a combination of genetic and environmental factors. Most CD-related genes are involved in mucosal bacterial recognition, secondary immune responses, and epithelial barrier integrity [1]. The major environmental factors are intestinal bacteria and foods that act as exogenous antigens [2]. A breakdown in tolerance, such as the hyperactivation of mucosal T cell responses against exogenous antigens,

leads to disease development [3]. The importance of food as an environmental factor is supported by the clinical finding that exclusion diets in CD patients achieve or maintain remission [4, 5]. Although the putative effects of nutritional therapies appear to be a result of bowel rest, provision of nutrients, alteration of bowel flora, or alteration of antigenic stimuli, the mechanism by which food antigens stimulate intestinal inflammation in CD remains unclear.

Immunoglobulin (Ig)Gs against microbial antigens, such as anti-*Saccharomyces cerevisiae* antibodies (ASCA) or dairy food antigens, are often detected in CD [6, 7], but there is little information regarding IgGs against a wider variety of foods. Moreover, the pathological reason for why IgG is detected in the serum of CD patients is unclear. Because T helper 1 cells (Th1) and T helper 17 cells (Th17) play an important role in CD inflammation [3], we hypothesized the food antigens to which IgG antibodies are produced in CD would induce an immune response and that elimination of these antigens might improve CD intestinal inflammation.

The objective of our study was to detect immune responses to food antigens in CD patients and to examine the mechanisms in a CD colitis mouse model. To elucidate the effect of foods as antigenic stimuli on the exacerbation of CD inflammation, we first compared the prevalence of IgG antibodies against food antigens in CD patients, UC patients, and healthy controls (HC). Second, we identified the antigenic food components and investigated the response of colitogenic T cells to food antigens using interleukin (IL)-10 knockout (KO) mice, which exhibit several characteristics of CD [8]. Finally, we investigated whether eliminating food antigens ameliorated colitis in mice transferred with IL-10 KO $CD4^+$ T cells.

Methods

Ethics statement

All human studies were conducted according to the principles of the Declaration of Helsinki and were approved by the institutional review boards of the Tokyo Yamate Medical Center (Tokyo, Japan) and Ajinomoto Co., Inc. (Kanagawa, Japan). Written informed consent was obtained from all patients. The Experimental Animal Ethics Committee of Ajinomoto Pharmaceuticals Co., Ltd. approved all animal procedures.

Subjects

Between September 2007 and April 2009, 98 Japanese patients with CD and 50 patients with UC attending the Tokyo Yamate Medical Center (formerly Social Insurance Chuo General Hospital) were enrolled in the study. Fifty-two healthy volunteers were also enrolled as HCs. The diagnoses of CD and UC were based on clinical, radiological, endoscopic, and histological criteria. The disease phenotype of CD patients (age at onset, disease location, and behavior) was determined according to the Montreal classification [9]. Each patient was assessed for perianal involvement and the presence of extraintestinal manifestations. The disease extent of UC patients was determined from the endoscopic findings. The disease activity was scored according to the International Organization for the Study of Inflammatory Bowel Disease (IOIBD) score in CD patients [10] and the Clinical Activity Index (CAI) in UC patients [11].

Serological analysis

Sera were collected during outpatient medical treatment at the hospital and analyzed for the presence of antibodies to foods. ASCA, a well-known antibody detected in 30–50 % of IBD patients [12, 13], was also analyzed to confirm that the cohorts in this study were appropriate subjects. IgGs to 88 foodstuffs (Table 2) were examined according to the IgG Food Antibody Assessment (Genova Diagnostics, Asheville, NC, USA), and the amount of IgG to each food was scored as 0, very low; 1, low; 2, moderate; or 3, high. Serum samples with a score of 2 or 3 were defined as positive. The number of IgG-positive foods in CD, UC, and HC participants was compared. The ASCA titer was determined using the ASCA IgG kit (Generic Assays, Dahlewitz, Germany). Serum samples whose antigen reactivity exceeded the cutoff range were considered positive.

Animals

IL-10 KO mice from a C57BL/6 background were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Mice backcrossed for ten generations with BALB/c mice were used in this study. Wild-type BALB/c mice were obtained from Charles River Japan (Tokyo, Japan), and CB-17/Icr SCID mice were obtained from Japan Crea (Tokyo, Japan). All mice were housed and bred under specific pathogen-free conditions at Pharmaceutical Research Laboratories, Ajinomoto Pharmaceuticals, Japan.

Immune response against food protein components in IL-10 KO mice

Serum was collected from 28- to 55-week-old IL-10 KO mice with colitis and 46-week-old BALB/c mice. Serum IgGs against food proteins were examined by enzyme

linked immunosorbent assay (ELISA). Food proteins from the NIH-07 standard diet (Oriental Yeast, Tokyo, Japan) (soybean, corn, wheat, fish meal, and skimmed milk) were extracted using phosphate buffered saline (PBS). Microtiter plates were coated with protein extracts and blocked with Immuno Block (DS Pharma Biomedical, Osaka, Japan). Serum samples from IL-10 KO or BALB/c mice were diluted in Immuno Block and then added to the wells in duplicate. Plates were incubated for 2 h at room temperature and then incubated for 1 h at room temperature with antibodies labeled with a horseradish peroxidase (HRP)-conjugated anti-mouse IgG-Fc fragment antibody. After the addition of 3,3′,5,5′-tetramethylbenzidine, the absorbance was measured at 450 nm.

Identification of food protein antigen

Protein was extracted from soybean, wheat, and corn with sample buffer containing 2 % sodium dodecyl sulfate (SDS) (w/v). Samples were heated at 95 °C for 5 min and centrifuged at 4 °C. The supernatant was analyzed by SDS–polyacrylamide gel electrophoresis (PAGE). Proteins were transferred to polyvinylidene fluoride membranes, which were blocked with 10 % non-fat dried milk. The membranes were blotted with IL-10 KO mouse serum, then with an HRP-conjugated secondary antibody, and detected using an ECL system (GE Healthcare, Tokyo, Japan) according to the manufacturer's instructions. Proteins in the detected bands were identified by peptide mass fingerprinting analysis (Protein Research Network, Kanagawa, Japan).

Preparation of fecal antigen extract

Fecal antigen extract was prepared as described previously [14]. Briefly, the colon and cecum were removed from BALB/c mice and placed in PBS with 10 μg/ml DNase and 1/5 volume glass beads (Sigma-Aldrich, MO, USA). The extract was sonicated three times for 30 s on ice and then centrifuged at 10,000×*g* for 10 min to remove insoluble material. The supernatant was collected, filter sterilized, and stored at −80 °C.

$CD4^+$ T cell isolation

IL-10 KO mice with colitis (displaying diarrhea and weight loss; 26–32 weeks of age, male, $n = 4$) and BALB/c mice (over 28 weeks of age, male, $n = 4$) were killed, and their mesenteric lymph nodes (MLN) were removed individually. $CD4^+$ T cells were positively isolated from single cell suspensions of MLNs using CD4 MicroBeads (Miltenyi Biotec, Bergisch-Gladbach, Germany).

Antigen-presenting cell (APC) preparation and $CD4^+$ T cell stimulation

APCs were prepared from BALB/c bone marrow according to previous methods [14]. Briefly, the femurs and tibiae were removed mechanically from the surrounding tissues, and the bone marrow cells were flushed with cold RPMI 1640 medium. Clusters within the marrow suspension were disassociated by vigorous pipetting, and erythrocytes were removed by hypotonic lysis. After filtration through a 70-μm nylon mesh, lineage-negative cells were isolated with the Lineage Cell Depletion Kit (Miltenyi Biotec). Depleted cells were cultured for 7 days in RPMI1640 supplemented with 10 % fetal bovine serum (FBS), 50 μM 2-mercaptoethanol, 10 ng/ml murine granulocyte–macrophage colony-stimulating factor (GM-CSF; R&D Systems, MN, USA), and 20 ng/ml murine IL-4 (R&D Systems). On day 7, non-adherent cells were collected by gentle pipetting, and dendritic cells (DCs) were isolated with CD11c MicroBeads (Miltenyi Biotec).

DCs were resuspended in RPMI 1640 medium, supplemented with 10 % FBS, 50 μM 2-mercaptoethanol and 10 ng/ml murine GM-CSF, and pulsed with 100 μg protein/ml of fecal extract, β-conglycinin (EPL Bio Analytical Services, IL, USA), or α-casein (Sigma). After overnight incubation, antigen-pulsed APCs were treated with mitomycin C (Kyowa Hakko Co, Ltd., Tokyo, Japan) and cocultured with $CD4^+$ T cells (1.5×10^4 APCs plus 5×10^4 $CD4^+$ T cells per well). In some experiments, an anti-MHC class II (I-A/I-E) monoclonal antibody (eBioscience, San Diego, CA, USA) or an isotype control antibody was added to the cell culture (2 μg/ml). Supernatants were collected after 5 days and frozen at −80 °C until required for cytokine measurements. Cytokines were measured using the BD CBA Mouse Th1/Th2/Th17 Cytokine Kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. Cell proliferation was measured from the metabolic activity using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). Assays for each condition were performed with $n = 4$. The results are shown as the mean ± SEM.

Mouse models of chronic colitis for evaluation of elimination diet feeding

Experimental diets were evaluated in a well-validated mouse model of colitis induced by adoptive transfer of IL-10 KO $CD4^+$ T cells. $CD4^+$ T cells were isolated from the spleens and MLNs of diseased IL-10 KO mice (21–35 weeks of age, male) and adoptively transferred to female C.B-17/lcr-scid/scid Jcl (SCID) mice (8 weeks of age, female) [15–17].

The powdered form of NIH-07 was used as the standard diet. The antigenic food protein components of NIH-

07 (soybean, corn, and wheat) were classified according to the presence of antibodies in IL-10 KO mice. The antigenic food protein components were eliminated from the standard NIH-07 diet, and soybean, an antigenic food protein, was added back at the level found in NIH-07. Casein and corn starch were added to make each diet isonitrogenic and isocaloric, respectively, to the NIH-07 diet. Mice were allowed free access to food and drinking water. Mice were killed for assessment 3 weeks after cell transfer, and colitis severity was assessed by measuring the colonic wet weight.

Histological scoring

Paraffin-embedded sections (2–3 μm) were stained with hematoxylin and eosin or periodic acid-Schiff. Histopathological analysis was performed with blinded scoring using the following criteria (modified from [18]): (1) degree of mononuclear cell infiltration in the lamina propria (score 0–3), (2) goblet cell disappearance (score 0–3), and (3) epithelial cell hyperplasia (score 0–3). The total histological score was calculated by combining the scores for each of the three parameters for a maximum score of 9.

Statistical analysis

All analyses were performed using the SAS statistical software package (version 8.02; SAS Institute Inc., Cary, NC, USA) or the EXSUS statistical software package (version 7.7.1; SAS Institute Inc.). Statistical comparisons were performed using an unpaired Student's t test, Mann–Whitney test, Tukey's multiple comparisons test, or Dunnett's test, as appropriate. Pearson's correlation coefficient analysis or Spearman's correlation coefficient analysis was used for correlation analysis. A probability value of $p < 0.05$ was considered statistically significant.

Results

Patient clinical characteristics

Patient and control demographics and clinical characteristics are shown in Table 1. The median age was similar in the UC, CD, and HC groups, and the median disease duration was longer in the CD group (10.0 years) than in the UC group (3.5 years). In the CD group, 87 % of patients had ileal lesions, and 67 % of patients had

Table 1 Subject characteristics

	CD ($n = 98$)	UC ($n = 50$)	HC ($n = 52$)
Female [n (%)]	26 (26.5)	26 (52.0)	22 (42.3)
Age[a] (years)	33.0 (18–68)	33.0 (18–70)	33.0 (22–52)
Disease duration[a] (years)	10.0 (1–28)	3.5 (0.5–25)	
Age at diagnosis[a] (years)	21.0 (0–54)	29.5 (13–65)	
Disease location [n (%)]	Ileal: 15 (15.3)	Proctitis: 16 (32.0)	
	Colonic: 13 (13.3)	Left-sided: 15 (30.0)	
	Ileocolonic: 70 (71.4)	Extensive: 19 (38.0)	
Disease behavior [n (%)]	Non-stricturing, non-penetrating: 14 (14.3)		
	Stricturing: 48 (49.0)		
	Penetrating: 36 (36.7)		
Perianal disease [n (%)]	77 (78.6)		
Past intestinal resection [n (%)]	66 (67.3)	2 (4.0)	
Disease activity score [n (%)]	IOIBD score	CAI score	
	0–1 points: 41 (41.8)	0–4 points: 40 (80.0)	
	2–4 points: 51 (52.0)	5–6 points: 8 (16.0)	
	5–7 points: 6 (6.1)	7–11 points: 2 (4.0)	
	8–10 points: 0 (0.0)	12 points: 0 (0.0)	
Treatment [n (%)]			
5-Aminosalicylate	90 (91.8)	50 (100)	
Prednisolone	8 (8.2)	9 (18.0)	
Azathioprine	41 (41.8)	3 (6.0)	
Infliximab	21 (21.4)	0 (0.0)	
Elemental diet	65 (66.3)	0 (0.0)	
Home IVH	3 (3.1)	0 (0.0)	

CD Crohn's disease, *UC* ulcerative colitis, *HC* healthy control, *IOIBD* International Organization for the Study of Inflammatory Bowel Disease, *CAI* Clinical Activity Index, *IVH* intravenous hyperalimentation

[a] Values are the median and range

Table 2 List of the 88 foods in the IgG Food Antibody Assessment and the percentage of CD patients, UC patients, and HCs with positive levels of IgG against the food items (*$p < 0.01$ vs. HC, **$p < 0.0001$ vs. HC; parametric Tukey's test)

	CD	UC	HC
Nuts, grains			
Almond	16*	4	0
Buckwheat	42**	2	2
Corn	67**	8	2
Corn gluten	11	0	0
Gluten	9	0	2
Kidney bean	10	4	10
Lentil	9	2	0
Lima bean	14*	0	0
Oat	45**	0	2
Peanut	13*	0	0
Pecan	38**	6	0
Pinto bean	20*	0	4
Rice	34**	2	0
Rye	3	0	0
Sesame	7	2	0
Soy	22**	2	0
Sunflower seed	11	0	0
Walnut	7	0	0
Wheat	5	0	0
Fish, shellfish			
Clam	12*	0	0
Cod	6	0	0
Crab	6	6	2
Lobster	6	2	0
Oyster	6	2	0
Red snapper	7	2	0
Salmon	8	4	2
Sardine	4	4	0
Shrimp	7	4	0
Sole	16	4	12
Trout	7	6	4
Tuna	12	6	4
Vegetables			
Alfalfa	27**	12	2
Asparagus	13*	2	0
Avocado	20*	0	0
Beets	17*	0	0
Broccoli	17*	2	0
Cabbage	48**	0	6
Carrot	12*	0	0
Celery	47**	4	0
Cucumber	17*	0	2
Garlic	7	2	0
Green pepper	26*	2	2
Lettuce	44**	4	2
Mushroom	7	2	0
Olive	4	0	0
Onion	22*	0	2
Pea	10	2	0
Potato, sweet	6	0	0
Potato, white	20*	0	0
Spinach	23**	0	0
String bean	19	8	13
Tomato	26**	0	0
Zucchini	10	0	0
Poultry, meats			
Beef	3	2	0
Chicken	6	4	0
Egg white	9	4	0
Egg yolk	13	12	4
Lamb	0	2	0
Pork	6	10	0
Turkey	4	8	0
Fruits			
Apple	11	2	0
Apricot	4	2	0
Banana	11	6	0
Blueberry	2	0	0
Cranberry	3	0	0
Grape	19*	10	2
Grapefruit	33**	0	0
Lemon	7	2	0
Orange	16*	0	0
Papaya	8	4	0
Peach	3	0	0
Pear	13	8	2
Pineapple	8	6	0
Plum	13	2	2
Raspberry	5	2	0
Strawberry	2	0	0
Dairy			
Casein	0	2	0
Cheddar Cheese	12	12	2
Cottage Cheese	8	8	2
Cow's milk	2	2	0
Goat's milk	0	0	0
Lactalbumin	0	0	0
Yogurt	1	2	2
Miscellaneous			
Yeast	53**	2	2
Cane sugar	52**	2	0
Chocolate	6	0	0
Coffee	6	2	0

Table 2 continued

	CD	UC	HC
Honey	3	2	0

The prevalence of the food items presented in boldface was higher than the prevalence of ASCA in CD patients

CD Crohn's disease, *UC* ulcerative colitis, *HC* healthy control

undergone operations for intestinal resection. In the UC group, the proportion of each proctitis type, left-sided and extensive, was about 30 %. Two patients with UC had undergone operations for total colectomy. In all, 71 % of CD patients and 24 % of UC patients had been treated with immunosuppressive drugs such as prednisolone, azathioprine, or infliximab. An elemental diet had been consumed as supplemental nutrition by 66 % of CD patients, and home intravenous hyperalimentation (IVH) had been administered in 3 % of CD patients because of short bowel syndrome. Most CD and UC patients (94 and 96 %, respectively) were in remission or had mild disease activity.

Seroreactivity to food antigens in IBD patients

We examined the levels of serum IgGs against 88 foods (Table 2) and determined the number of food items that scored 2 or 3 in each group. The number of IgG-positive foods was significantly larger in CD patients than in UC patients or HCs (Fig. 1a). CD patients had seroreactivity against an average of 12.7 foods [range 0–62; 95 % confidence interval (CI) 9.9–15.6], whereas UC patients and HCs demonstrated seroreactivity against an average of 2.3 foods (range 0–27; 95 % CI 1.1–3.5) and 0.9 foods (range 0–5; 95 % CI 0.5–1.3), respectively. The percentage of participants with positive levels of IgG against vegetables, grains, and nuts, but not dairy and meats, was significantly higher among CD patients than among UC patients and HCs. In particular, over 50 % of CD patients had significantly higher levels of IgG against corn, yeast, or cane sugar. Although ASCA was also detected in 30 % of CD patients, 11 foods (corn, yeast, cane sugar, cabbage, celery, oat, lettuce, buckwheat, pecan, rice, and grapefruit) induced higher IgG sensitivity than ASCA (Table 2). The ASCA IgG titer did not correlate with the number of IgG-positive food items (Fig. 1b).

Of the clinical characteristics, younger age and some inflammatory markers, such as high white blood cell count, high platelet count, high serum amyloid A protein level, low serum albumin level, and high CRP level, correlated with the greater number of IgG-positive food items in CD patients (Supplementary Table 1A, 1B). There were no significant correlations between the number of IgG-positive foods and other factors, such as patient gender, disease location, disease behavior, disease activity, and history of medical or surgical treatment (Supplementary Table 1B).

Seroreactivity to food antigens in IL-10 KO mice

Next, to elucidate the immunological mechanisms through which food antigens affect intestinal inflammation in CD, we evaluated the effects of food antigens in a murine CD colitis model. We analyzed the levels of serum IgG antibodies against food protein components of the standard NIH-07 diet (soybean, corn, wheat, fish meal, skimmed milk) in IL-10 KO mice (Fig. 2a). Higher levels of antibodies to soybean, corn, and wheat protein were detected in IL-10 KO mice, when compared to levels in control mice. However, similar levels of antibodies to milk and fish meal protein were present in IL-10 KO mice and BALB/c control mice. IgG antibodies against the foods were also detected specifically in colitic IL-10 KO mice (Fig. 2b).

Response of colitogenic T cells to food antigens

We hypothesized that colitogenic T cells are activated by food antigens via antigen-presenting DCs. To test this, antigenic proteins were detected. The proteins detected in a peptide mass fingerprinting analysis of soybean, wheat, and corn are shown in Fig. 3a. Western blotting of purified β-conglycinin revealed the presence of serum IgG against β-conglycinin in IL-10 KO mice (Fig. 3b). Using ELISA, we confirmed that the levels of serum IgG against β-conglycinin were equivalent to the levels of serum IgG against soybean (Fig. 3c).

The response of T cells to food antigens was assessed using β-conglycinin as an antigenic food protein, α-casein from skimmed milk as a non-antigenic food protein, and fecal extract as a positive control. Each antigen was presented on DCs prepared from BALB/c bone marrow and co-cultured with $CD4^+$ T cells isolated from IL-10 KO mice or BALB/c mice. The T cell proliferative response induced by fecal extract or β-conglycinin was higher than that induced by α-casein in IL-10 KO cells. However the proliferative responses in IL-10 KO cells were equivalent to those in BALB/c cells (Fig. 4a). In $CD4^+$ T cells from IL-10 KO mice, the IFN-γ (Fig. 4b) and IL-17 (Fig. 4c) production induced by β-conglycinin or fecal extract was higher than that induced by α-casein. Cytokine production in response to β-conglycinin or α-casein was lower in the $CD4^+$ T cells of BALB/c control mice than in the cells of IL-10 KO mice. IL-4, a Th2 cytokine, was not detected after stimulation with β-conglycinin or α-casein (data not shown). The addition of an anti-MHC class II antibody inhibited proinflammatory cytokine production by $CD4^+$ T cells in response to β-conglycinin (Fig. 4d, e).

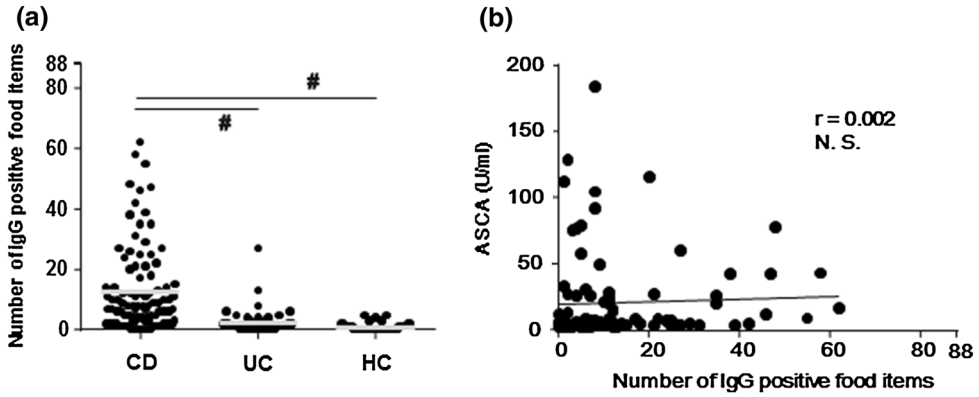


Fig. 1 High seroreactivity to food antigens in Crohn's disease patients. **a** The number of IgG-positive food items for each subject is indicated by a *dot*, and the average is indicated with *red horizontal bars*. Statistics: p values were adjusted for multiple comparisons using the non-parametric Tukey test. $^{\#}p < 0.0001$ for CD vs. UC and for CD vs. HC. **b** Correlation of ASCA IgG (U/ml) and the number of IgG-positive food items in the plasma of Crohn's disease patients. Statistics: Pearson's correlation coefficient analysis was performed, $R^2 = 0.002150$, $p = 0.6503$

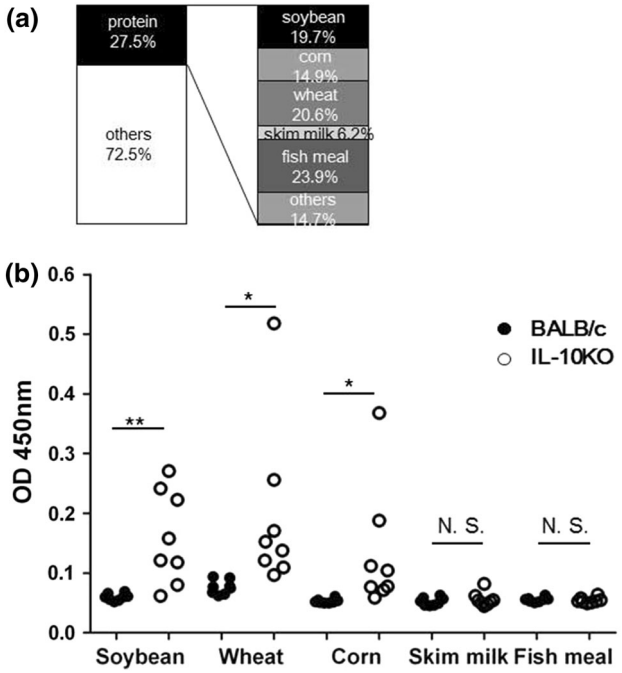
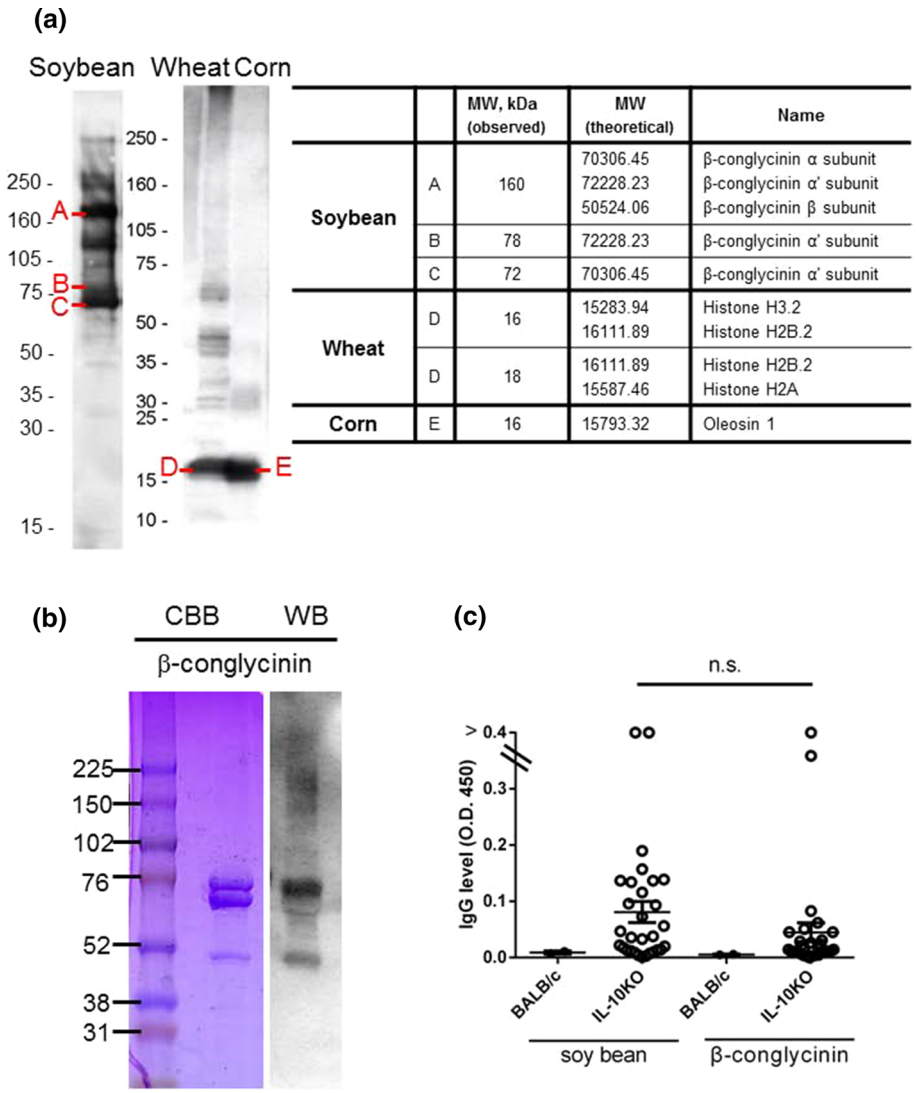


Fig. 2 IL-10 KO mice as a murine colitis model exhibit seroreactivity to food proteins. Serum IgGs against soybean, corn, and wheat proteins were detected in IL-10 KO mice but not in control mice. **a** Protein components of NIH-07. **b** Levels of serum IgG antibodies against food proteins found in NIH-07. *Black circle*: BALB/c mice; *open circle*: IL10-KO mice. Statistics: $*p < 0.05$, $**p < 0.01$ vs. BALB/c mice; *t* test

Elimination of food antigens ameliorates the development of colitis in mice transferred with IL-10 KO $CD4^+$ T cells

We examined whether T cells activated by food antigens exacerbated colitis. $CD4^+$ T cells derived from IL-10 KO mice, including T cells with reactivity to food antigen, were transferred into SCID mice to induce colitis, and the effect of antigen removal was assessed. An elimination diet was prepared by eliminating the food components from NIH-07 for which antibodies were specifically present in IL-10 KO mice (soybean, corn, and wheat). The feeding experiment was performed as shown in Supplementary Figure 1A. There was no significant difference in food intake in any of the groups (data not shown). As shown in Supplementary Figure 1B and 1C, mice transferred with IL-10 KO cells and fed the standard NIH-07 diet had significantly higher colon weights (586 ± 13 mg vs. 191.3 ± 4.6 mg; $p < 0.001$) and higher histological scores than non-transferred control mice fed the standard NIH-07 diet. Mice transferred with IL-10 KO cells and fed the elimination diet had significantly lower colon weights (354 ± 16 mg vs. 586 ± 13 mg; $p < 0.001$) and lower histological scores than transferred mice fed the standard NIH-07 diet (Supplementary Figure 1B and 1C). To investigate the effect of changes in diet composition on the microbiota, we analyzed the fecal microbiota of mice transferred with IL-10 KO cells using terminal restriction fragment length polymorphism (T-RFLP) analysis (Supplementary Figure 1D). The cluster analysis was based on the similarity of the T-RFLP profiles for *Msp*I and *Hha*I digestion. The microbiotas of three mice with colitis fed NIH-07 and seven mice without colitis fed the elimination diet clustered together. Thus, eliminating antigenic food components did not normalize the microbiota towards that found in healthy normal mice.

To evaluate the effect of antigenic food protein on colitis development, we re-introduced soybean, which contains β-conglycinin, into the elimination diet (Fig. 5a). When the antigenic food was added to the elimination diet, histological score and colon weight increased (Fig. 5b–d). Weight loss associated with colitis was observed in



		MW, kDa (observed)	MW (theoretical)	Name
Soybean	A	160	70306.45	β-conglycinin α subunit
			72228.23	β-conglycinin α' subunit
			50524.06	β-conglycinin β subunit
	B	78	72228.23	β-conglycinin α' subunit
	C	72	70306.45	β-conglycinin α' subunit
Wheat	D	16	15283.94	Histone H3.2
			16111.89	Histone H2B.2
	D	18	16111.89	Histone H2B.2
			15587.46	Histone H2A
Corn	E	16	15793.32	Oleosin 1

Fig. 3 β-conglycinin α identified as a food protein antigen from soybean in IL-10 KO mice. **a** Western-blotting analysis of food components in NIH-07 with serum IgG from IL-10 KO mice (*left*) and the protein mass fingerprinting analysis of the detected proteins (*right*). **b** Western-blotting analysis of purified β-conglycinin using serum IgG from IL-10 KO mice. **c** Levels of serum IgG against soybean and β-conglycinin in IL-10 KO mice

transferred mice fed the control diet, but not in transferred mice fed the elimination diets, with or without added antigen, indicating that there were no nutritional problems with the elimination diet compositions (Fig. 5e).

Discussion

This study showed that CD patients have a markedly increased immune response to food antigens, as reflected by the high levels of serum IgG against many food types, when compared to UC patients and HCs. In this study, 30 % of the CD patients were sensitive to ASCA, similar to previous findings (30–50 %) [13] This suggests that our cohort of CD patients was typical and did not possess an unusually high hyper-immune response.

Does the degree of antigen exposure in the daily diet influence the production of antibodies against various foods in CD patients? In this study, two-thirds of the CD patients who consumed an elemental diet also produced serum IgG antibodies against many types of food, and there were no significant differences in the number of IgG-positive food items when CD patients with an elemental diet were compared to those without an elemental diet (Supplementary Table 1B). However, in patients undergoing IVH, the number of the antibody-positive food items was low, and no increase was found in follow-up a half year later. In addition, although the number of IgG-positive food items did not correlate with the amount of elemental diet consumed daily, among the patients with a high elemental diet intake, there were a few cases in which the number of the antibody-positive food items increased at follow-up approximately 6 months later (Supplementary Figure 2). Previous dietary intervention studies have also shown that CD patients react to the exclusion of various food antigens identified by serum IgG or IgG4 [19–21]. These findings suggest that daily exposure to food antigens induces high sensitivity to many types of foods in CD patients.

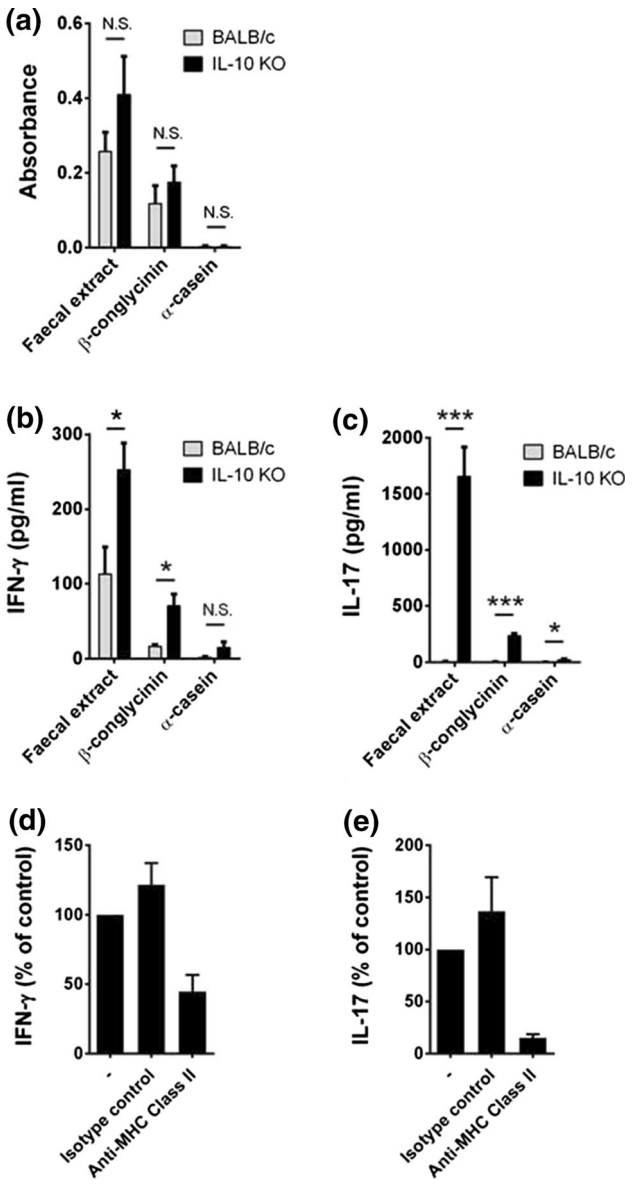
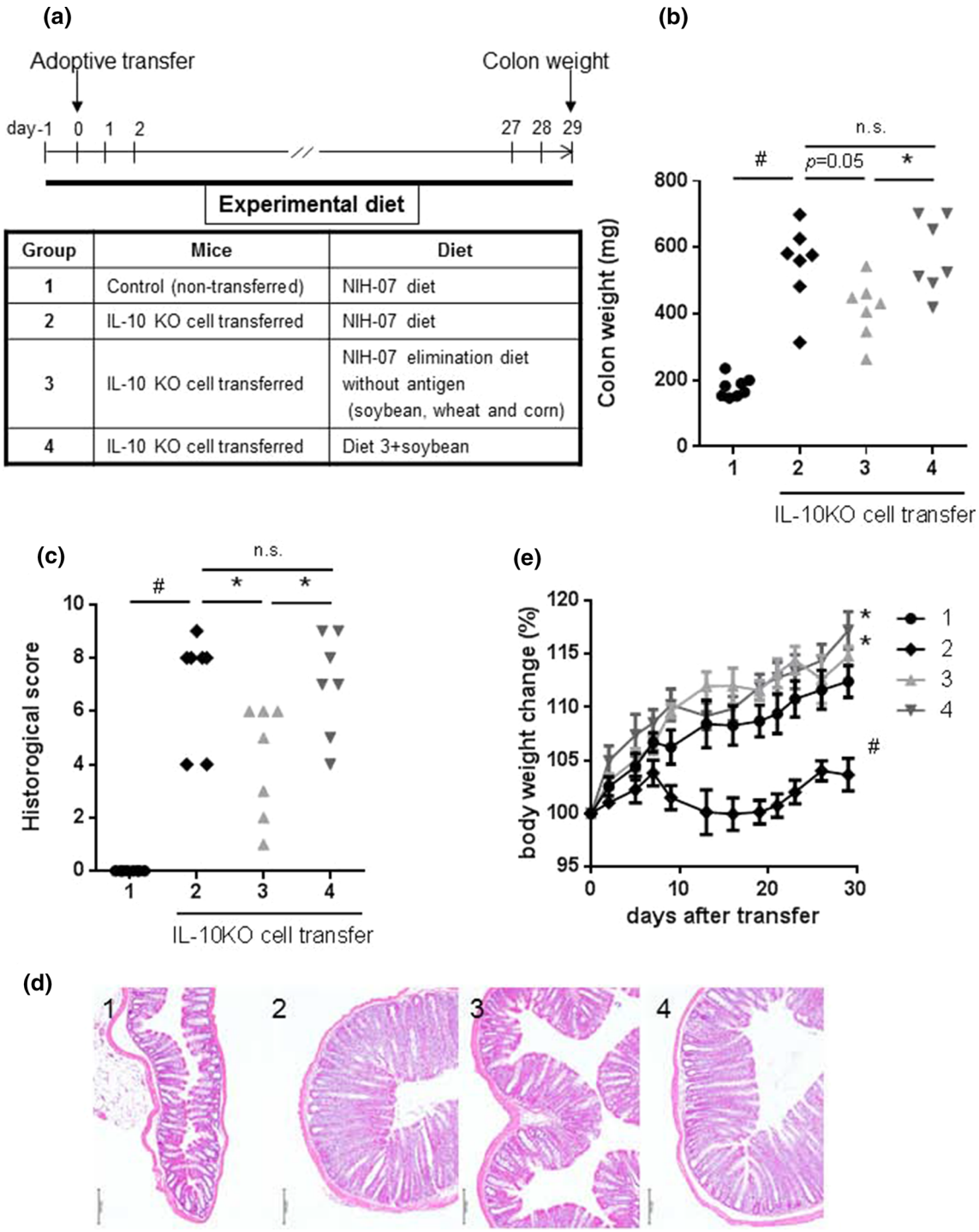


Fig. 4 Colitogenic $CD4^+$ T cells from IL-10 KO mice are activated by antigenic food protein-primed DCs through MHC class II. β-conglycinin and α-casein were selected as antigenic and non-antigenic food proteins, respectively. Fecal extract was used as a positive control. **a** Cell proliferation of $CD4^+$ T cells. **b**, **c** Th1 and Th17 cytokine production by $CD4^+$ T cells. **d**, **e** Effect of an anti-MHC class II antibody on the $CD4^+$ T cell response to β-conglycinin

To establish that food antigen exposure affects food sensitivity, it would be best to test whether a protein antigen stimulates $CD4^+$ T cells in the human intestinal tract. However, we used mice because there were technical difficulties in identifying the proteins that acted as food antigens in humans and in testing human gut immunity. We found that food antigens associated with high IgG levels activated Th1/Th17, which are considered important for the onset of colitis. Furthermore, in the IL-10 KO cell-transfer mice, in which $CD4^+$ cells were transferred into a SCID mouse, the food-antigen elimination diet attenuated colitis. Adding antigenic food back into the elimination diet exacerbated colitis. That fact that B cells were not present and that antibody was not produced in IL-10 KO cell-transfer mice indicates that IgGs to foods do not directly affect the exacerbation of enteritis; rather, it appears that T cells activated by food antigens are important. Van den Bogaerde [22] showed that responses to food antigens in CD patients can lead to increased proliferation of peripheral blood lymphocytes. In contrast, Brimnes [14] observed that $CD4^+$ T cells did not respond to DCs pulsed with food pellet extracts. However, it is possible that the amount of antigenic food protein presented to DCs was insufficient, or that only some of the proteins in the chow were antigenic. In our experiments, we used a single food antigen, β-conglycinin, but we assume that antigenic proteins from other foods that were antigenic in our study would also activate $CD4^+$ T cells through DCs. Further intervention studies are necessary to investigate which food proteins are antigenic in humans and whether antigenic food proteins stimulate $CD4^+$ T cells in CD patients. From a practical standpoint, an elemental diet rather than an elimination diet might be better able to prevent food antigen intake.

Our study showed that the number of IgG-positive foods correlated with early onset and inflammatory status. Many reports have shown that patients with active CD, especially pediatric patients, have increased intestinal permeability. [23–25] An impaired barrier in association with active and repetitive inflammation increases the exposure of the mucosal innate and acquired immune system to luminal antigens such as food antigens. This could be a trigger for the increase in the number of IgG-positive foods. In addition, the antibody titers to diet were higher in CD patients than in UC patients, likely because of hyperpermeability of the small intestine in which most of food proteins were absorbed. In UC patients, it is thought that the exposure of food antigens in the large intestine with hyperpermeability is little, because the function of digestion and absorption of food proteins in the small intestine is normal. In CD patients, the number of IgG-positive food items did not vary depending on disease location (Supplementary Table 1B), a result which might have been influenced by the following: the number of the patients with large intestine-type disease was small (13 %), and even patients with large intestine-type disease might have hyperpermeability of the small intestine.

Do immunosuppressive drugs inhibit responses to food antigens? In this study, although the ratio of immunosuppressive drug use was higher in CD patients than in UC patients, the immune response to food antigens was higher in the CD group. In addition, the number of IgG-positive food items did not vary depending on the administration of immunosuppressive drugs in the CD group. However, the impact of immunosuppressive drugs on food antigen–



Group	Mice	Diet
1	Control (non-transferred)	NIH-07 diet
2	IL-10 KO cell transferred	NIH-07 diet
3	IL-10 KO cell transferred	NIH-07 elimination diet without antigen (soybean, wheat and corn)
4	IL-10 KO cell transferred	Diet 3+soybean

Fig. 5 Elimination of food antigens ameliorates the development of colitis and the re-addition of food antigens induces colitis in mice transferred with IL-10 KO cells. **a** Experimental design for elimination diet feeding in mice transferred with IL-10 KO cells. **b** Colon weight on day 29 ($^{\#}p < 0.001$, *t* test vs. group 1; $*p < 0.05$, Tukey's multiple comparisons test). **c** Histological score on day 29 ($^{\#}p < 0.001$, Mann–Whitney test vs. group 1; $*p < 0.05$, Tukey's multiple comparisons test). **d** Histology sections on day 29. *Scale bars* 200 μm. **e** Body weight change after IL-10 KO cell transfer ($^{\#}p < 0.01$ vs. group 1; $*p < 0.01$ vs. group 2, Dunnett's test)

antibody reactions was not determined because this study was not an intervention study with immunosuppressive drugs.

Our study showed a high prevalence of IgG against grains and yeast. These results correspond with the results reported by Bentz et al. [20] from a study of CD patients in Germany; however, the low prevalence of IgG against cheese (8–12 %) or wheat (5 %) described in this study does not correspond with the results of the previous study. Although it has not been

established whether milk protein has antigenicity in CD patients [7, 26], in our study, dairy foods rarely showed antigenicity in CD patients. Differences in the dietary habits of the study participants at the different study locations could account for the discrepancies. Interestingly, the pattern of immune responses in IL-10 KO mice, in which soybean, corn, and wheat acted as antigens while other food proteins such as fish meal and skimmed milk did not, was similar to the pattern of immune responses in CD patients.

Although nutrient composition usually affects the microbiota, the microbiotas of IL-10 KO cell-transferred mice fed the NIH-07 diet and IL-10 KO cell-transferred mice fed the NIH-07 antigen-elimination diet clustered in the same group (Supplementary Figure 1). This suggests that the suppression of colitis exacerbation was not due to an extensive change in the microbiota, but was due to a reduction in the interaction between antigenic food protein-presenting DCs and $CD4^+$ T cells.

Conclusions

Both CD patients and CD colitis mice showed high seroreactivity against many food antigens. In the CD colitis mice, intestinal inflammation via $CD4^+$ T cell hyperactivation was induced by food antigens associated with high serum IgG levels and was ameliorated by the elimination of food antigens. In CD patients, whether disrupted immunological tolerance to food antigens acts as an exacerbating factor remains to be determined. Further studies, such as those investigating the response of $CD4^+$ T cells to food antigens and the effectiveness of eliminating food antigens, are required to prove that food antigens contribute to the pathogenesis of human CD.

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