Published in final edited form as:

Inflamm Bowel Dis. 2008 December ; 14(12): 1641–1651. doi:10.1002/ibd.20645.

Enhanced CBir1-specific innate and adaptive immune responses in Crohn's disease

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Abstract

CBir1 is a dominant antigen with a role in innate and adaptive immunity in mouse models of colitis and antibodies to CBir1 are associated with severe human Crohn's disease. Our aim was to determine whether CBir1 stimulates innate and antigen-specific T-cell responses in Crohn's disease. We demonstrate that CBir1 enhanced IL-6 and IL-1 β production by peripheral blood (PB) monocytes. IL-6 was significantly increased in monocytes from Crohn's disease compared to controls and ulcerative colitis. Anti-CBir1⁺ patients and IL-6 was inversely correlated. A significant increase in CBir1 specific peripheral T cell proliferation was more evident in cells from Crohn's disease than controls and ulcerative colitis. CBir1 induced increased numbers of IFN- γ ⁺ cells in lamina propria mononuclear cells (LPMC) from Crohn's disease compared to ulcerative colitis and controls. CBir1 induces enhanced peripheral innate, and peripheral and mucosal antigen-specific T-cell responses in Crohn's disease. Consistent with results from the mouse, CBir1 immune activation could play a role in Crohn's disease.

Keywords

Adaptive Immune Systems in IBD; Animal Models of IBD; Innate Immune Systems in IBD; Inflammation in IBD. T Cells and Regulatory T Cells in IBD

Introduction

Crohn's disease (CD) is a chronic inflammatory bowel disease that may involve any part of the gastrointestinal tract. There is increasing evidence that genetic, environmental and immunological factors are involved in the pathogenesis, but the precise etiology and pathogenic mechanisms remain unknown. The prevailing hypothesis is that CD is the result of an aberrant immunological response to gut commensal bacteria in genetically susceptible individuals.^{1, 2} Furthermore, it is the altered interplay between the innate and adaptive immune systems' response to these organisms that results in chronic intestinal inflammation.^{3, 4}

The surface of the human intestine is densely colonized by a variety of largely commensal microbial species.⁵ Flagellin is an interesting candidate for a role in mucosal immune responses. It is a structural protein of the flagellum, a surface filament required for bacterial motility. In pathogenic bacteria, flagella and chemotaxis machinery contribute to virulence by providing motility towards niches where infection can take place.⁶ Specifically, flagellin

is a common bacterial antigen present on most motile bacteria in the gut.⁷ How flagellated bacteria physiologically deliver signals to the host is unknown. It may be shed from the bacterial surface by host proteases or detergents such as bile salts or surfactants. Sensing flagellin by TLR5 may act as an early detection system that triggers a rapid host response.^{8, 9}

The importance of flagellin-mediated TLR5 signalling in mucosal inflammation has been shown in animal studies. In spontaneously colitic mice, a random identification found that 25% of antigenic targets of serum antibodies were flagellins.¹⁰ CBir1 flagellin-generated CD4⁺ Th1 and Th17 cell lines can induce colitis when transferred into SCID mice by reactivity to endogenous CBir1 flagellin in the microbial flora.^{10, 11} In addition to T-cell mediated injury, flagellin can induce inflammation by activation of innate immune responses. In the DSS-induced mouse colitis model, intra-colonic administration of flagellin aggravates the inflammation.¹² Sanders *et al* also showed that following an i.p. injection with flagellin, flagellin-specific Ig generation required activation of innate immunity in that these responses did not develop in MyD88-deficient mice. In addition, flagellin from *Helicobacter pylori*, which is known not to activate TLR5, also did not elicit antibodies.¹³

CBir1 is a novel bacterial flagellin discovered by serologic expression cloning from spontaneously colitic C3H/HeJBir mice.¹⁰ This is the first antigen to be linked between mouse and human and to be shown to have a direct pathogenic effect in the development of T-cell-mediated colitis in mice.^{10, 11} The mouse cytokine deficiency-induced colitis susceptibility (Cdcs) 1 locus was recently identified and found to reduce NFκB1-driven cytokine release from bone-marrow derived macrophages and dendritic cells.¹⁴ The Cdcs1 allele defect in innate immunity as measured by bone marrow derived dendritic cell IL-6 production was associated with an exaggerated adaptive T cell proliferation and IFN-γ immune response to CBir1.¹⁴

Our previous study in humans showed that serum anti-CBir1 antibodies were detected in approximately half of CD patients.¹⁵ Anti-CBir1 was independently associated with distinct phenotypic manifestations of CD. Our group recently expanded on these observations and showed that anti-CBir1 serum activity in CD was independently associated with fibrostenosis (FS) and complicated small bowel (SB) CD.¹⁶ The importance of serum antibody reactivity to microbial antigens becomes even more interesting in view of data suggesting a link with disease course. Patients with high serum reactivity to microbial antigens (including CBir1 flagellin) have an aggressive disease course.^{17–19}

These findings provide evidence that CBir1 flagellin is an immunodominant antigen of the enteric microbial flora. The expression of antibodies to CBir1 is indicative of an adaptive immune response to this antigen. As mentioned above, CBir1 is the first antigen discovered to be related to both animal and human IBD pathogenesis as evidenced by antigen specific T cell responses, and that in animals have been demonstrated to be a colitis-causing antigen. The role of CBir1 flagellin stimulation of innate immune activation and induction of Th1 responses in CD patients is unknown. Therefore, the aim of this study was to investigate the relationship of CBir1 to both innate and adaptive immune responses in CD patients and compare these to ulcerative colitis (UC) and normal controls.

Materials and Methods

STUDY POPULATION

Patients with CD or UC were selected randomly from the patient population of the Cedars-Sinai Inflammatory Bowel Disease Center, and randomly assigned for cytokine (IL-6 and IL-1β) analysis, T cell proliferation assay, C-reactive protein (CRP) and erythrocyte

sedimentation rate (ESR). Samples from 25 CD, 17 UC patients and 12 healthy controls underwent monocyte induced cytokine analysis. Another 21 CD, 15 UC patients and 12 healthy controls were studied in the T cell proliferation assay. A separate group of sera from 8 CD patients was used to assess antigen specificity of the T cell response. In total, we studied 54 CD patients and 32 UC patients. The diagnosis for each patient was based on standard endoscopic, histologic and radiographic features. Phenotype analysis was made by different investigators using definitions of clinical subgroups previously reported.¹⁷ The control group is a collection of healthy adults with no symptoms/signs of disease. Investigators were blinded, unaware of disease phenotype, activity and anti-CBir1 serum level. Disease location and treatment regimens of the study populations are listed in Tables 1A and 1B.

Intestinal specimens were obtained from patients undergoing surgical resection of the colon for treatment-resistant UC or Crohn's colitis at Cedars-Sinai Medical Center, Los Angeles, CA. In this study, all tissue specimens were taken from a macroscopically involved or uninvolved area of resected colon when available. Non-IBD colonic resection specimens were also collected as control. Lamina propria mononuclear cells (LPMCs) were isolated using a technique modified from that described previously.³²

All research-related activities were approved by the Cedars-Sinai Medical Center Institutional Review Board.

PREPARATION OF CBir1

Recombinant CBir1 was prepared as described previously.¹⁰ Briefly, CBir1 was expressed by transforming plasmid DNA into BL21 pLysS competent cells (Novagen, San Diego, CA) and inducing a single colony cell culture with 2mmol/L isopropyl- β -thiogalactopyranoside (Sigma, St. Louis, MO). Recombinant protein was recovered from cell lysate with Ni-NTA Agarose beads (Qiagen, Valencia, CA) by following the manufacturer's instructions and was dialyzed in 10mmol/L Tris (pH 4 to 11), depending on the predicted recombinant pI characteristics. Recombinant proteins were quality-checked for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by staining with Coomassie blue and by N-terminal protein sequencing, and were quantified with a Micro BCA assay (Pierce, Rockford, IL). Recombinant proteins were assayed for endotoxin contamination with the Limulus assay (Bio Whittaker, Walkersville, MD).

PERIPHERAL BLOOD MONOCYTE ISOLATION AND CULTURE

PBMCs were isolated on standard Ficoll-Hypaque density gradients. Subsequent isolation of monocytes was performed using the Monocyte Isolation Kit II (Miltenyi Biotec, Auburn, CA) according to the manufacturer's protocol. Monocyte preparations were routinely >90% pure as determined by esterase stain (Sigma-Aldrich). Isolated monocytes were adjusted to 1×10^6 cells cultured in 1 ml RPMI 1640 containing 2 mM glutamine and 25 mM HEPES buffer (Mediatech, Herndon, VA) supplemented with 10% Fetal Bovine Serum (FBS), 50 μ g/ml gentamicin (Omega Scientific, Tarzana, CA), and 0.25 μ g/ml amphotericin B (Gemini Bioproducts, Woodland, CA).

REAL-TIME PCR ANALYSES

Total RNA was isolated from monocytes using TRIzol (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. 500 ng of total RNA was used in each real-time reaction, with oligo(dT) as primer, using the Omniscript kit and protocol (Qiagen, Valencia, CA). IL-1 β , IL-6 and β -actin transcripts were amplified by quantitative real-time PCR with TaqMan probes and primers designed using Beacon Design 4.0 (Premier Biosoft International, Palo Alto, CA). PCR was done on 1/4 the real-time reaction in

duplicate as follows: 50°C for 2 min, 95°C for 2 min, then 50 cycles at 95°C for 15 s, and 60°C for 1 min. Assays were performed following the predeveloped TaqMan assay reagents protocol for Platinum qPCR mix (Invitrogen Life Technologies) in an iCycler (Bio-Rad, Hercules, CA). The iCycler Optical system Interface (Bio-Rad) was used to analyze samples. Duplicates differing by less than one cycle were averaged and amount of transcript was analyzed as 2E for each sample and expressed as percentage of β -actin. The sequence of the primer are listed as follows: IL-1 β (forward: TATAGCCTGGACTTTCCTGTTGTC; reverse: GCTGACTGTCCTGGCTGATG; probe: 5'-56-FAM/ACACCAATGCCCAACTGCCTGCCT-3' BHQ_1), IL-6 (forward: CTTCCGGTCCAGTTGCCTTCTC; reverse: AAGAGGTGAGTGGCTGTCTGT; probe: 5'-56-FAM/CTGCTCCTGGTGTGCCTGCTGCC-3' BHQ_1) and β -actin (forward: GACTACCTCATGAAGATCCTCACC; reverse: TCTCCTTAATGTCACGCACGATT; probe: 5'-FAM/CGGCTACAGCTTCACCACCACGGC-3' BHQ_1).

ENZYME-LINKED IMMUNOSORBENT ASSAYS

Enzyme-linked immunosorbent assay analysis of anti-CBir1 was performed as previously described¹⁴ without knowledge of diagnosis or other serology results. Briefly, enzyme-linked immunosorbent assay plates were coated overnight with 1 μ g/well of CBir1, and then blocked with 1% bovine serum albumin in phosphate-buffered saline for 2 hours. Plates were washed and serum was added at a 1:200 dilution in 1% bovine serum albumin-phosphate-buffered saline for a 30-minute incubation. After washing, horseradish-peroxidase-conjugated anti-human IgG at a 1:10000 dilution was added and incubated for 30 minutes. After another wash, the plates were incubated with tetramethylbenzidine substrate for 15 minutes. The reaction was stopped with 1 N sulfuric acid and read at 450 nm. Positive was defined as the mean $\pm$ 2 SD of the healthy controls.

T CELL PROLIFERATION ASSAY

PBMCs were isolated as described previously. Subsequent isolation of CD4⁺ T cells was performed using the CD4⁺ T Kit (Miltenyi Biotec) according to the manufacturer's protocol. A fraction of PBMCs were irradiated (33 Gy) and adjusted to 1×10^6 cells in 1 ml RPMI 1640. Irradiated PBMCs were respectively pulsed with CBir1 (10 μ g/ml) or tetanus (control, 1:1000, Public Health Biologic Lab, Boston, MA) for 16 hours. Cells were washed, counted and mixed with autologous CD4⁺ T (20×10^3) cells at a ratio of 1:1 in 200 μ l of medium in a round-bottom 96-well plate (Nunc, Rochester, NY) for 4 days. In MHCII blocking experiment, pulsed PBMCs were first cultured with anti-HLA-DR mAb (10 μ g/ml, G46-6, BD Biosciences, San Diego, CA) or its isotype control (10 μ g/ml, eBM2a, eBiosciences, San Diego, CA) for 30 mins at room temperature. PHA (control, 2 μ g/ml) was also added in one of the conditions. During the last 16 h of the culture period, the cells were loaded with 1 μ Ci/well [³H] thymidine (ICN Biomedicals, Costa Mesa, CA). The mean counts per minute (CPM) of triplicate wells were calculated.

ISOLATION OF LAMINA PROPRIA MONONUCLEAR CELLS

Briefly, the intestinal specimen was washed with Hank's Balanced Salt Solution (HBSS) and the mucosa was dissected away from the underlying layers. The mucosa layer was incubated in a shaking water bath (100 rpm) in calcium- and magnesium-deficient HBSS, containing 1 mM EDTA, 50 μ g/ml gentamicin, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 50 μ g/ml amphotericin B, with the solution changed every 30 min until the supernatant was free of epithelial cells. The remaining LP was minced into 1- to 2-mm pieces and was digested for 10 min in RPMI 1640 containing 10% FCS, 0.5 mg/ml collagenase B (Boehringer Mannheim, Gaithersburg, MD), 1 mg/ml hyaluronidase (Sigma-Aldrich), 0.1 mg/ml DNase I (Sigma-Aldrich), 50 μ g/ml gentamicin, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 50 μ g/ml fungizone, in a shaking water bath (100 rpm). The

supernatants was collected, filtered through 110- μ m nylon mesh (Spectrum Laboratory Products, Gardena, CA), and centrifuged at $500 \times g$ for 5 min. The cell pellet was resuspended in 15 ml of solution and was centrifuged at $30 \times g$ for 5 min to remove epithelial and other large cells. The supernatant was removed, and lymphocytes were isolated by separation on Ficoll-Hypaque gradients. The cells were then washed three times with HBSS and resuspended in complete RPMI.

QUANTIFICATION OF CBIR1-SPECIFIC T CELLS BY ELISPOT ASSAY

The frequency of CBir1-specific IFN- γ -releasing T cells in LPMCs was determined using a direct *in vitro* enzyme-linked immunospot (Elispot) assay. 4×10^5 LPMCs in 200 ml RPMI 1640 were respectively cultured with or without CBir1 (10 μ g/ml) or tetanus (1:1000) overnight at 37°C/5%CO₂ in a round-bottom 96-well plate. A positive control well with 20×10^3 LPMCs with PMA and ionomycin (P/I) at a final concentration of 5 μ g/ml respectively were included. 96-well dynex microlite plate (Fisher) was precoated with anti-human IFN- γ capture monoclonal antibody (5 μ g/ml; BD-Pharmingen) overnight at 4°C in borated buffered saline (BBS). Plates were first washed three times with 200 ml/well PBS/Tween, and then blocked with 0.5% BSA/PBS for 30 mins at room temperature. Cell culture was transferred to the microlite plate and incubated for 3 days at 37°C/5%CO₂. Wells were again washed 3 times with PBS/Tween and a biotinylated anti-human IFN- γ antibody (BD-Pharmingen, 0.5 μ g/ml in 0.5% BSA/PBS) was added in 100 μ l aliquots, and the plates were incubated for 2 h at room temperature. Subsequently the plates were washed 4 times with PBS/Tween and then incubated with HRP coupled streptavidin (1:1000 diluted in 0.5% BSA/PBS, Jackson, Bar Harbor, ME) for 30 mins at room temperature. A further three washes with PBS/Tween were performed before addition of 100 μ l/well enhanced DAB solution (Pierce). Color development occurred within 15 mins at room temperature. Plates were further washed 2 times with distilled water and were air dried. Spots were initially observed and counted under a dissection microscope.

STATISTICAL ANALYSIS

Statistical analysis was performed with Prism[®] Mac 3.0. Differences between various groups were tested with nonparametric (Mann-Whitney test) statistics for quantitative levels. To determine the associations between anti-CBir1 antibody responses to cytokine production (IL-1 β and IL-6), univariate analyses using χ^2 tests were performed. The Wilcoxon matched pairs test (nonparametric test) was conducted to identify differences between paired groups. A *P* value (*P* trend) of .05 or less suggests that the linear trend was statistically significant.

Ethical Considerations—None of the authors has conflicts of interest pertaining to this manuscript. Human subject participation was in accordance with the regulations established and enforced by the Cedars-Sinai Institutional Review Board.

Results

CBIR1-INDUCES INCREASED LEVELS OF IL-6 IN PERIPHERAL MONOCYTES FROM CD PATIENTS COMPARED TO THOSE WITH UC AND CONTROL SUBJECTS

C3H/HeJBir mice carrying the colitogenic Cdc1 allele developed severe colitis in the absence of IL-10, and showed reduced innate immune responses (IL-6, IL-1 β , IL-12) to CBir1 but an increased CD4 T cell response (IFN- γ and T cell proliferation). Colitis in C57BL/6 mice (colitis resistant) was exacerbated after Cdc1 translocation,¹⁴ which indicates that the gene(s) within the Cdc1 region impairs the innate immune response to CBir1 and in turn skews the adaptive immune response toward compensatory hyperresponsiveness and chronic intestinal inflammation. The first goal of this study was to determine the effect of CBir1 on human innate immune responses in CD patients. To

accomplish this goal, monocytes were isolated from PB and cocultured with CBir1 for 4 hours at which time mRNA was isolated. To parallel mouse studies, IL-6 and IL-1 β mRNA were measured using real-time PCR and values were normalized to β -actin expression. CBir1 induced both IL-6 and IL-1 β mRNA from monocytes from healthy donors, and patients with UC and CD (Figure 1A and 1B). To evaluate whether CBir1-induced upregulation of IL-6 and IL-1 β is greater among CD patients, we further normalized values to their basal level. As shown in Figure 1C, we found that CBir1 induced a significantly higher amount of IL-6 in monocytes from CD patients than healthy donors and UC (Figure 1C, healthy: 1.4 ± 0.4 ; CD: 6.3 ± 1.7 and UC: 1.7 ± 0.3). In contrast, there was no significant increase in IL-1 β (Figure 1D, healthy: 59.0 ± 25.1 ; CD: 122.2 ± 50.6 and UC: 81.9 ± 13.8). Therefore, CBir1 induces significantly higher levels of IL-6 compared to UC and healthy donors.

IL-6 PRODUCTION AND HIGH ANTI-CBIR1 LEVEL ARE INVERSELY CORRELATED IN CD

Antibodies to CBir1 represent an adaptive immune response to this antigen. In animal studies, colitogenic mice had decreased innate immune associated IL-6 production and an enhanced T cell proliferative and IFN- γ adaptive response to CBir1.¹⁴ Although there was an overall increase in IL-6 induction within the CD population, there was a degree of variation. To test the relationship of the level of innate to adaptive responses in a given CD patient, we correlated the level of CBir1 induced IL-6 mRNA to the level of anti-CBir1. Although a higher level of anti-CBir1 was associated with 50% of CD patients, “Low” anti-CBir1 (<25 μ g/ml) responses are found in most of the remaining 50% of CD patients as well as patients with UC and healthy subjects.¹⁵ We therefore selected patients with “medium” and “high” (>30 μ g/ml) anti-CBir1 expression to determine any correlation with IL-6 and IL-1 β , respectively. Anti-CBir1 ELISA measurement and correlation analyses were done in a “double-blinded” manner without knowledge of cytokine level. As shown in Figure 2A, in CD patients with anti-CBir1 level >30 μ g/ml, we found an inverse correlation between IL-6 and anti-CBir1 ($R^2 = 0.2526$, $p < 0.05$). For IL-1 β , even though there was no significant increase in an overall CD population (Figure 1D), we observed a similar but not a significant inverse relationship between levels of IL-1 β and level of antibodies to CBir1 (Figure 2B). These data are consistent with the recent observations by Beckwith and colleagues in mice, which show a lower induction of IL-6 from bone marrow derived DC and an increase in adaptive CBir1 specific T cell responses.¹⁴

CBIR1 INDUCES ENHANCED PB T CELL PROLIFERATION IN CD

In contrast to a lower innate immune response in colitis, Beckwith *et al* showed an enhanced memory proliferative T cell response to CBir1.¹⁴ In order to investigate the immune effect of CBir1 in the adaptive immune system, we tested PB T cell proliferation in CD. PB T cells were isolated and cocultured with CBir1 or tetanus pulsed APC for 4 days, [3 H] thymidine was added and the mean CPM of triplicate wells was calculated. In CD patients a significant increase from baseline in CBir1 mediated peripheral T cell proliferation was observed compared to control and tetanus (Figure 3A, control: 760.9 ± 341.4 ; CBir1: 4524 ± 998.1 and tetanus: 2413 ± 994.3 , control vs. CBir1: $p < 0.001$; tetanus vs. CBir1: $p < 0.05$). To evaluate whether the T cell response was CD specific, we also measured the proliferation in UC and healthy donors (Figure 3B). We observed that CBir1 specific T cell proliferation was higher in CD patients (4524 ± 998.1) compared to those from healthy donors (1453 ± 207.8 , $p < 0.05$) and UC (2425 ± 1073 , $p < 0.05$). There was no significant difference between UC and healthy donors. In contrast, though tetanus slightly enhanced T cell proliferation, there is no difference among healthy donors, UC and CD (Figure 3B).

To directly evaluate whether CBir1 is a specific antigen in CD, we blocked HLA mediated CBir1 antigen presentation in the proliferation assay. As shown in Figure 3C, anti-HLA-DR

mAb completely abrogated CBir1-induced T cell proliferation ($p < 0.01$, *vs. controls*). In contrast, isotype control was unable to inhibit proliferation. These data demonstrate that CBir1 is a specific antigen that triggers T cell proliferation in CD.

Increased proliferation in response to both CBir1 and tetanus in CD patients might represent a “global” T cell hyperresponsiveness reaction rather than a more selective CBir1-mediated T cell response. Therefore, we paired the CBir1 and tetanus data from the same CD patient to evaluate whether T cell proliferation was more CBir1-dominant. There was no association between CBir1 and tetanus (Figure 3D, $R^2 = 0.3685$, $p = 0.43$). These data indicate that CBir1 mediated T cell proliferation is more dominant in CD. Because anti-CBir1 is also a measurement of adaptive immune responses, we also correlated T cell proliferation to anti-CBir1 level. Results showed that T cell proliferation seen in CD is independent of anti-CBir1 level (anti-CBir1 level $30 \mu\text{g/ml}$, $n=13$, Figure 3E, $R^2 = 0.009$, $p = 0.67$).

THE NUMBER OF CBIR1 SPECIFIC IFN- γ -PRODUCING CELLS IS INCREASED AMONG LPMCS FROM CROHN'S DISEASE COLON

To evaluate whether a similar increased CBir1 mediated T cell response as seen in PB cells was present in the mucosa, we measured the number of IFN- γ producing cells in LPMCs isolated from CD, UC and normal surgical specimens. IFN- γ has been used to measure mouse and human mucosal T cell responses for global and antigen specific activation. LPMCs were isolated and incubated with and without CBir1 in an Elispot assay for 4 days. As shown in Figure 4A and B, CBir1 induced an elevated number of IFN- γ^+ producing cells in LPMCs from CD (non-involved: 20.8 ± 3.4 , $p < 0.05$; involved: 26.5 ± 4.1 , $p < 0.01$, *vs. controls*), compared to 11.6 ± 2.8 from UC (*n.s.*, *vs. controls*) and 9.8 ± 2.1 from controls. LPMCs from disease-involved mucosa contain more IFN- γ producing cells than LPMCs from uninvolved specimens (Figure 4C, $p < 0.05$). These data demonstrate that an increased number of CBir1 responsive T cells exist in the mucosa as well as the peripheral immune compartment in CD patients.

Discussion

The CBir1 biological effect in mice has been studied in detail, however, its effect on the peripheral and mucosal immune responses in CD patients remains unknown. In this study we demonstrate that CBir1 induces enhanced innate and specific peripheral and mucosal T cell responses in CD as compared to UC or healthy donors. When anti-CBir1 level is used as a measure of an adaptive immune response to CBir1 within CD, those with the highest anti-CBir1 response have the lowest induction of IL-6 and IL-1 β .

In our study, the activation of the NF- κ B pathway was assessed by measuring NF- κ B-dependent IL-6 and IL-1 β . NF- κ B is an attractive candidate not only because its deletion in mice is associated with spontaneous and *Hepaticus*-induced colitis,²⁰ but also because the NF- κ B signaling pathway activates many proinflammatory cytokines, adhesion molecules, chemokines, and metalloproteinases that contribute to inflammation.²¹ Furthermore, several other target genes for the p50/p65 heterodimer are inflammatory in nature (IL-12 p40 and TNF). It is possible that the differences in IL-6 expression induced by CBir1 could represent differential expression of TLR5 between CD and UC or within different populations of CD patients. Experiments to determine the relative role of differential receptor expression *vs.* post receptor function are needed, but for the present, the possibility of doing so is limited by the number of cells obtainable from patients. It is not clear why we found higher levels of IL-6 but not IL-1 β in CD. It might be due either to a limited kinetic analysis of IL-1 β mRNA expression or a better correlation with protein expression. In addition, although the association between CBir1 expression and IL-1 β does not quite reach statistical significance, there is a similar inverse correlation with level of antibodies to CBir1. The inverse

correlation of IL-6/IL-1 β and anti-CBir1 antibody is consistent with the reduced induction of NF- κ B driven inflammatory genes that is associated with the colitogenic Cdc1 locus in the mouse.¹⁴ Our findings showed that in patients with CD, blunted NF- κ B activation (lower IL-1 β and IL-6) is associated with a compensatory adaptive immune response (higher anti-CBir1 antibody). These results suggest that a defective innate immune response may fail to clear CBir1 associated organisms, resulting in an enhanced adaptive Th1 response systemically and within the mucosa leading to mucosal inflammation.

In support of the relationship between anti-CBir1 expression and lower IL-6 induction, and its relationship to NF- κ B expression, we have shown that an anti-CBir1-associated NF- κ B gene haplotype (H1) is associated with decreased activation of NF- κ B,²² consistent with the former observation of colitogenic Cdc1 locus in the mouse.¹⁴ This observation supports the notion that genetic variation determines response to CBir1 flagellin in this region in both mouse and human and raises the possibility that genetic variation in the NF- κ B gene creates an abnormal innate response to CBir1 that results in a more severe CD.

In this context, how effective TLR5 and NOD2 signaling affect CBir1 flagellin recognition and the outcome of CD remains to be established. It is of note that patterns of immune responses to bacterial antigens among CD patients differ from those of animal models. CBir1 clearly is not the only bacterial antigen involved in the pathogenesis of CD. In contrast, in the mouse a single bacterium could induce experimental colitis in germ-free environments,^{23, 24} and a relatively selective serum and T-cell immune response in a given animal model indicates a limited number of bacterial antigens.^{25, 26} In addition, TLR5 knockout mice were found recently to develop rather than protect from spontaneous colitis.²⁷ It has been indicated that switching-off TLR5 expression will alternatively enhance TLR4 expression in the mucosa and therefore cause the onset of inflammation. At least in this study, the discrepancy might be due to TLR5 activation in mucosa, which usually does not occur as a global mucosal signaling but remains localized to limited areas where infection takes place. Further studies should correlate CBir1-TLR5 signalling to their inflammation-inducing capacity. A detailed intercellular pathway analysis including MyD88 protein, NOD2 and NF- κ B (and/or MAPK) is needed in patients with CD.

As previously shown by Lodes *et al*, adoptive transfer of CBir1-specific T cells induced severe colitis in C3H/HeJBir mice,^{10, 11} and CD4⁺ T cells responded to CBir1 but not other flagellins as evaluated by T cell proliferation assay. Our data from humans indicated that peripheral and mucosal T cell clones are activated by CBir1, and this activation was enhanced in CD compared to UC or healthy donors. These T cell proliferation data suggest that CBir1 might be an immuno-dominant antigen that stimulates a subset of pathogenic T cell responses in a subset of CD patients. Unlike IL-6, no relationship was observed in T cell proliferation with anti-CBir1. This may well be due to the limited number of samples examined with high level of anti-CBir1. However, if true, the reasons for this discrepancy will need to be examined in further studies.

The effect of medication in this study could be ruled out. A subgroup analysis (data not shown) among anti-TNF, 5-ASA, steroid, immunosuppressant treated and non-treated CD patients showed no significant differences in IL-6, IL-1 β level, nor in T cell proliferation. In addition, there was no relationship with disease location (data not shown). The previous study by Lodes *et al* also showed in mice that an increased titer of anti CBir1 correlated positively with worsening IBD histopathology,¹⁰ therefore anti-CBir1 level could be used for evaluating disease activity. To address the same issue in CD, we correlated immune response with disease activity. Although lacking statistical significance (data not shown), we found elevated IL-6, IL-1 β and CBir1-specific T cell proliferation in “active” (CRP: 0.8 $\pm$ 0.3) CD compared to “inactive” (CRP: 0.2 $\pm$ 0.1). These data are consistent with the

observations in mice, which show an increased CBir1 specific immune responses. Future studies sequentially assessing T cell responses in the same CD patient while active and in remission will be required to formally confirm this association.

In addition to CBir1 induction of peripheral T cell activation, we observed elevated numbers of IFN- γ secreting cells in LPMCs isolated from CD specimens that were cultured with CBir1. Elevated IFN- γ may up-regulate the expression of major histocompatibility complex DR antigens on epithelial cells,²⁸ and also increase NOD2 and TLR expression resulting in increased susceptibility to commensal bacterial structures, such as LPS.²⁹ These end results of IFN- γ signalling could be part of the complex pathophysiology of barrier disruption as is observed in IBD. Because IFN- γ is capable of up-regulating macrophage IL-12 production,³⁰ an enhanced IL-12/IFN- γ (Th1) loop is likely to be involved in perpetuating chronic inflammation which originated from CBir1. It is of note that, though Th1 cells have been widely accepted as playing a key role in colitis development, Th17 cells have recently been identified as a lineage distinct from Th1 and Th2 cells that may play a pivotal role in intestinal inflammation.³¹ A commensal flagellin-specific Th17 cell clone has been identified and has been shown to induce severe colitis in mice.¹¹ Therefore, whether CBir1-specific Th17 cells play a role in the pathogenesis of CD will be important to investigate in future studies.

One might speculate from this study that an aberrant monocyte/macrophage and T cell response to CBir1 contributes to the mucosal inflammation in CD. Determining the basis for selective responses to CBir1 present in the normal commensal flora of most individuals is a challenging proposition. Data are emerging (but are not yet definitive) to suggest there are subpopulations of CD who respond to this antigen. As an example, our previous study¹⁵ showed that there are specific subsets of patients with CD that display patterns of seroreactivity against CBir1 and have a more severe clinical outcome. The results in our current study are consistent with this finding, as up-regulation of IL-6 and T cell proliferation were present in only half of the CD patients.

Taken together, the present data demonstrate for the first time that CBir1 enhances the IL-6/IL-1 β /IFN- γ production and thus Th1-like cytokine responses in CD. As demonstrated in animal models, T cells reacting to CBir1 cause disease in mice and the studies described above show for the first time in humans that enhanced specific T-cell reactivity against CBir1 was found in a subset of patients with CD compared to patients with UC or in controls. Given the activity of flagellin as a specific ligand for TLR5, these data provide a potentially important link between adaptive and innate immune responses in mucosa in the pathogenesis of IBD. Our findings together with the others, support the hypothesis that CD is a combined result of a defect in tolerance to bacterial antigens in those with an underlying genetic susceptibility. Among patients with CD, immune responses to CBir1 may be related to different pathophysiologic mechanisms and may represent distinct genotypes and phenotypes. Investigations are underway to identify specific functional consequences of genetic variations in patients who express high levels of anti-CBir1.

Acknowledgments

This work was supported by USPHS/NIDDK grants P01 DK071176 and P01 DK046763.

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Figure 1 A&B

Figure 1 C&D

Figure 1.

CBir1 enhanced IL-6 and IL-1 β production of PB monocytes.

Blood (healthy: 12, CD: 25 and UC: 17) was collected as described in *Materials and Methods*. Monocytes were isolated and cultured for 4 hours with CBir1. IL-6 (A) and IL-1 β (B) mRNA expression measured in real-time PCR is presented as a ratio after normalization to the housekeeping gene β -actin. (C) and (D), mRNA expression are shown after normalization to its basal level. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s. no significant difference).

Figure 2.

IL-6 mRNA inversely correlated with level of anti-CBir1 (≥ 30 $\mu\text{g/ml}$).

Monocyte isolation and cytokines measurement (CD: 15) are explained previously in the legend for Figure 1. Anti-CBir1 was measured by ELISA as described in *Materials and Methods*. Samples with anti-CBir1 level lower than 30 $\mu\text{g/ml}$ (10 samples) are not included in this figure. Correlations between IL-6 (A) and IL-1 β (B) mRNA by level of anti-CBir1 (≥ 30 $\mu\text{g/ml}$) in given CD patients are shown respectively.

Figure 3 A

Figure 3B

Figure 3C

Figure 3 D&E

Figure 3.

Enhanced CBir1 generated PB T cell proliferation in CD.

A: Irradiated human PBMCs were used as APCs (stimulator cells) and autologous CD4⁺ T cells were used as responder cells (healthy: 16, CD: 21 and UC: 15). Irradiated APCs were pulsed with CBir1 or tetanus and mixed with autologous CD4⁺ T cells as described in *Materials and Methods*. PHA, as a positive control, was added in one of the conditions. In the HLA DR blocking experiment, pulsed PBMCs were first cultured with anti-HLA-DR mAb or its isotype control for 30 mins at room temperature. During the last 16 h of the culture period, the cells were loaded with 1 μ Ci/well [³H] thymidine. The mean CPM of triplicate wells was calculated. CPM: counts per minute (* $p<0.05$, *** $p<0.001$, n.s. no significant difference). B: increased CBir1-specific PB T cell proliferation in CD compared

to UC and healthy donors. C: HLA blockage completely abrogates CBir1 mediated T cell proliferation in CD patients (n=8). D: no correlation between CBir1 and tetanus-stimulated T cell proliferation in CD patients (n=21). E: no correlation between CBir1-stimulated T cell proliferation by level of anti-CBir1 ($30 \mu\text{g/ml}$) in given CD patients (n=13).

Figure 4. Increased CBir1 specific IFN- γ -producing cells in LPMCs from involved CD colon. Colonic surgical specimens (control: 10, UC: 12, CD_{un}: 10 and CD_{inv}: 9) were collected. LPMCs and Elispot conditions were prepared as described in *Materials and Methods*. Color development and spots were observed and triplicate wells were counted. The mean number

of triplicate wells was calculated. A: a representative picture of IFN- γ Elispot assay from a CD patient (*Left*: PMA and ionomycin, 20×10^3 LPMCs; *Middle*: Non-treated, 1×10^6 LPMCs; *Right*: CBir1, 1×10^6 LPMCs). B: an increased amount of CBir1 specific IFN- γ -producing cells (4×10^5 LPMCs) from CD colon compared to UC and control. C: an increased amount of IFN- γ -producing cells from CD involved colon compared to uninvolved. CD_{un}: uninvolved CD colon; CD_{inv}: involved CD colon (* $p < 0.05$, ** $p < 0.01$, n.s. no significant difference).

Table 1

A) Disease Location

	CD				UC		
	Ileum ^b	Ileum/Colon	Colon	Total	Left-sided	Pan-colitis	Total
Cytokine Assay (IL-6/IL-1 β)	11(44)	13(52)	1(4)	25(100)	5(29)	12(71)	17(100)
Proliferation Assay	5(24)	11(52)	5(24)	21(100)	2(13)	13(87)	15(100)

^aPatients were selected randomly. Investigators were blinded, unaware of disease phenotype and activity. The diagnosis for each patient was based on standard endoscopic, histologic and radiographic features. Phenotype analysis was made by different investigators using definitions of clinical subgroups previously reported.¹⁷ Data are shown as number of patients and its percentage.

^bIleum (colon), lesions were found only in ileum (colon); Ileum/colon: in both ileum and colon. Right side, cecum, ascending and transverse colon; Left side, descending colon, sigmoid and rectum; Pan colitis, including both right and left side.

Table 1

B) Therapy of the Patients^a

	Cytokines assay (IL-6/IL-1 β)		Proliferation assay		Elispot assay (surgical specimen)		
	CD	UC	CD	UC	UC	CD _{non}	CD _{inv}
5-ASA	9(36)	7(41)	8(38)	7(47)	0(0)	0(0)	0(0)
Steroid	5(20)	6(35)	4(19)	5(33)	7(58)	4(40)	6(67)
Immunosuppressant	13(52)	4(24)	8(38)	4(27)	4(33)	2(20)	4(44)
Anti-TNF	4(16)	0(0)	8(38)	3(20)	1(8)	2(20)	2(22)
Antibiotics	0(0)	1(6)	3(14)	2(13)	0(0)	0(0)	0(0)
NT ^b	5(20)	7(41)	3(14)	5(33)	4(33)	5(50)	2(22)
TOTAL ^c	25(100)	17(100)	21(100)	15(100)	12(100)	10(100)	9(100)

^aPatients were selected and phenotype analysis was made as described previously in table (A). Data are shown as number of patients and its percentage.^bNT: Non-treatment^cPart of the patients were treated more than one category of drug.