

BASIC AND TRANSLATIONAL—ALIMENTARY TRACT

Commensal-Specific CD4⁺ Cells From Patients With Crohn's Disease Have a T-Helper 17 Inflammatory Profile



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BACKGROUND & AIMS: Crohn's disease (CD) has been associated with an altered immune response to commensal microbiota, mostly based on increased seroreactivity to microbial proteins. Although T cells are believed to contribute to the development of CD, little is known about the antigens involved. We investigated the antigen-specificity of T cells isolated from patients with CD. **METHODS:** We isolated peripheral blood mononuclear cells from 65 patients with CD and 45 healthy individuals (controls). We investigated T-cell reactivity to commensal microbial antigens using proliferation assays (based on thymidine incorporation and carboxyfluorescein succinimidyl ester dilution). Gene expression patterns were determined using microarray and real-time polymerase chain reaction analyses. Cytokines, chemokines, and antibodies were measured by enzyme-linked immunosorbent assay, flow cytometry, or multiplex cytokine assays. Intestinal crypts were obtained from surgical resection specimens of 7 individuals without inflammatory bowel disease. We examined the effects of commensal-specific CD4⁺ T cells on primary intestinal epithelial cells from these samples. **RESULTS:** The bacterial proteins FlaX, A4-fla2, and YidX increased proliferation of CD4⁺ T cells isolated from peripheral blood of patients with CD compared with controls. In blood samples from controls, CD4⁺ T cells specific for FlaX, A4-fla2, or YidX had a T-helper (Th)1 phenotype; a larger proportion of CD4⁺ T cells specific for these proteins in patients with CD had a Th17 phenotype or produced Th1 and Th17 cytokines. When supernatants collected from commensal-specific CD4⁺ T cells from patients with CD were applied to healthy intestinal epithelial cells, the epithelial cells increased the expression of the chemokine (C-X-C motif) ligand 1 (CXCL1), CXCL8 and the CC chemokine ligand 20 (CCL20). **CONCLUSIONS:** A larger proportion of commensal-specific CD4⁺ T cells from patients with CD have a Th17 phenotype or produce Th1 and Th17 cytokines, compared with T cells from controls; this might contribute to intestinal inflammation in patients with CD. These cells might be targeted for treatment of CD. The transcriptional data of commensal-specific CD4⁺ T cells from healthy individuals and CD patients have been deposited in the Gene Expression Omnibus at the National Center for Biotechnology Information (accession no: GSE70469).

Keywords: Antigen-Specific Immune Response; IBD; Immunity; Effector T Cells.

Crohn's disease (CD) is a chronic remitting and relapsing inflammatory disease of the intestinal tract that is thought to result from a loss of tolerance to commensal microorganisms. This view is supported strongly by studies conducted in animal models of intestinal inflammation, in which pathogenic CD4⁺ T-cell responses are directed against the enteric microbiota.^{1–4}

In CD patients, antibodies to several microbial components have been detected in serum, indicating an exacerbated acquired immune response toward commensal microbiota. These antibodies recognize microbial components such as *Saccharomyces cerevisiae* oligomannan (anti-*Saccharomyces cerevisiae* antibodies [ASCA]),⁵ *Escherichia coli*'s outer membrane protein C (OmpC),⁶ or various subtypes of flagellins such as A4-fla2, FlaX, FliC, or CBir1.^{7–9} The search for novel bacterial-reacting immunoglobulins in the serum of CD has led recently to the identification of new *E coli*-derived seroreactive proteins such as YidX, FrvX, Era, and GabT.¹⁰ Importantly, seroreactivity to microbial antigens correlates with complicated disease,^{3,11,12} encouraging the exploitation of these antibodies as predictors of disease course.^{13–15}

Although the presence of antimicrobial antibodies in CD patients⁶ strongly suggests that a microbial-specific T-helper response is generated as well, little evidence has

Abbreviations used in this paper: Ag, antigen; ASCA, anti-*Saccharomyces cerevisiae* antibody; CD, Crohn's disease; CFSE, carboxyfluorescein succinimidyl ester; IBD, inflammatory bowel disease; IFN, interferon; IL, interleukin; PBMC, peripheral blood mononuclear cell; r-IL, recombinant interleukin; TCR, T-cell receptor; Th, T-helper; TT, tetanus toxoid; UC, ulcerative colitis.

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been found to support the existence of a T-cell response toward bacterial antigens in CD patients.¹⁶ It generally is accepted that T cells, and more specifically CD4⁺ T cells, play a pathogenic role in CD because they heavily infiltrate involved areas of the intestinal mucosa and extensive data from experimental models support this.¹⁷ These cells show T-helper (Th)1 and Th17 proinflammatory profiles.^{18–22} Despite being regarded as pathogenic key players in CD, their antigen specificity remains largely unexplored. Most of the studies that have focused on T-cell reactivity to the intestinal microbiota used total bacterial sonicates or bacterial protein pools to stimulate T cells. Although these data showed that T cells from CD patients overreacted to intestinal bacteria, no specific antigens were identified.^{23–25} More recently, the group of Targan,¹⁶ based on findings in animal models of inflammatory bowel disease, were able to detect CBir1-reactive T cells in CD patients.

Defining an antigen-specific, T-cell response in the context of CD would have many implications. From a pathophysiological point of view, it would reinforce the concept of loss-of-tolerance toward commensal organisms. Moreover, it will enable exploring the nature and function of such cells. From a clinical point of view, it also may provide a possible mechanism for disease relapse and damage progression, as well as potential new specific therapeutic targets.

We therefore set out to investigate the T-cell response to several commensal bacterial- and yeast-derived proteins in CD patients. Our data provide evidence of the existence of commensal microbial-specific CD4⁺ T cells in healthy individuals and in CD patients. More importantly, we show that although commensal-specific CD4⁺ T cells from healthy controls show a Th1 phenotype, these cells present a unique Th17-biased transcriptional and functional profile in CD patients and they are able to amplify the inflammatory response in the intestinal epithelium. Based on these findings, we hypothesize that specifically targeting these microbial-reacting T cells could represent a novel and effective approach to CD treatment.

Materials and Methods

More detailed information is provided in the online [Supplementary Materials and Methods](#) section.

Study Subjects

Patients diagnosed with CD (N = 65) by endoscopic, histologic, and radiologic criteria were recruited for the study. Healthy volunteers (N = 45) without any known underlying acute or chronic pathologic condition served as control donors. Epithelial crypts were obtained from surgical resection specimens from non-inflammatory bowel disease (IBD) individuals (N = 7) undergoing surgery for colorectal cancer; the healthy mucosa was separated from the lesion by at least 10 cm. Demographic and clinical characteristics are summarized in [Supplementary Table 1](#). This study was approved by the Ethics Committee at the Hospital Clínic de Barcelona and was performed in accordance with the principles stated in the Declaration of Helsinki. All patients, healthy controls, and non-IBD subjects signed an informed consent before their inclusion in the study.

Antigen Stimulation of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized peripheral blood by Ficoll (Sigma-Aldrich, Madrid, Spain) gradient centrifugation. For T-cell proliferation screening, PBMCs were cultured with the microbial commensal antigens ASCA-Ag (*Saccharomyces Cerevisiae* oligomannan; antigen recognized by ASCA), CBir1, FlaX, A4-fla2, and YidX (Prometheus Laboratories, Inc, San Diego, CA) at 1 µg/mL for 7 days. ³H thymidine (1 µCi/well; Amersham, Cambridge, United Kingdom) incorporation took place during the last 16 hours of culture. For major histocompatibility complex II blocking, anti-HLA-DR, -DP, and -DQ (BD Biosciences, Franklin Lakes, NJ) was added at 20 µg/mL to the culture. For carboxyfluorescein succinimidyl ester (CFSE) (Cell Trace CFSE cell proliferation kit; Thermo Fisher Scientific, Waltham, MA) staining, PBMCs were incubated with CFSE at 5 µmol/L and cultured with tetanus toxoid (TT; Sigma-Aldrich) and microbial commensal antigens (Prometheus Laboratories, Inc) all at 2 µg/mL for 7 days.

Cytokine Analysis

For intracellular cytokine staining, cells were restimulated with phorbol myristate acetate and ionomycin in the presence of brefeldin A (all from Sigma-Aldrich) for the final 4 hours of culture. Cells were fixed and permeabilized with FIX and PERM (Thermo Fisher Scientific). Cells were stained with a Live/Dead fixable violet dead cell stain kit (Thermo Fisher Scientific) and fluorescent antibodies. Cells were acquired in a Fluorescence-Activated Cell Sorting Canto II (BD Biosciences) and analyzed with BD Fluorescence-Activated Cell Sorting Diva Software v6.1.1.

Cytokine secretion by PBMCs was measured by an enzyme-linked immunosorbent assay or using the multiplex cytokine assay Milliplex Human Th17 magnetic bead (EMD Millipore Corporation, Billerica, MA).

Cell Sorting of Antigen-Specific CD4⁺ T Cells and RNA Isolation

CFSE-labeled PBMCs were cultured in the presence of TT, FlaX, A4-fla2, or YidX antigens. Recombinant interleukin (IL)2 (20 IU/mL) was added to the culture on day 7. Viable CFSE⁺ CD4⁺ cells were sorted on day 14 of culture in a Fluorescence-Activated Cell Sorting Aria II (BD Biosciences) and restimulated with antigen in the presence of autologous irradiated PBMCs. Ten days later RNA was extracted.

Intestinal Crypt Isolation and Culture

Non-IBD intestinal epithelial crypts were isolated from intestinal tissue as previously described.²⁶ For short-term crypt culture, 30 isolated crypts/25 µL Matrigel (BD Biosciences) were plated and cultured in either complete crypt culture medium or in medium containing supernatants from activated sorted antigen-specific CD4⁺ T cells. Antigen-specific T-cell supernatants were obtained from restimulating sorted cells with anti-CD3 (BD Biosciences) and anti-CD28 (BD Biosciences) at 1 µg/mL for 5 days. Neutralization of IL17 was achieved by preincubation of T-cell supernatants with monoclonal anti-IL17A (R&D Systems, Minneapolis, MN). After overnight culture of crypts, RNA was extracted. Chemokine analysis in culture

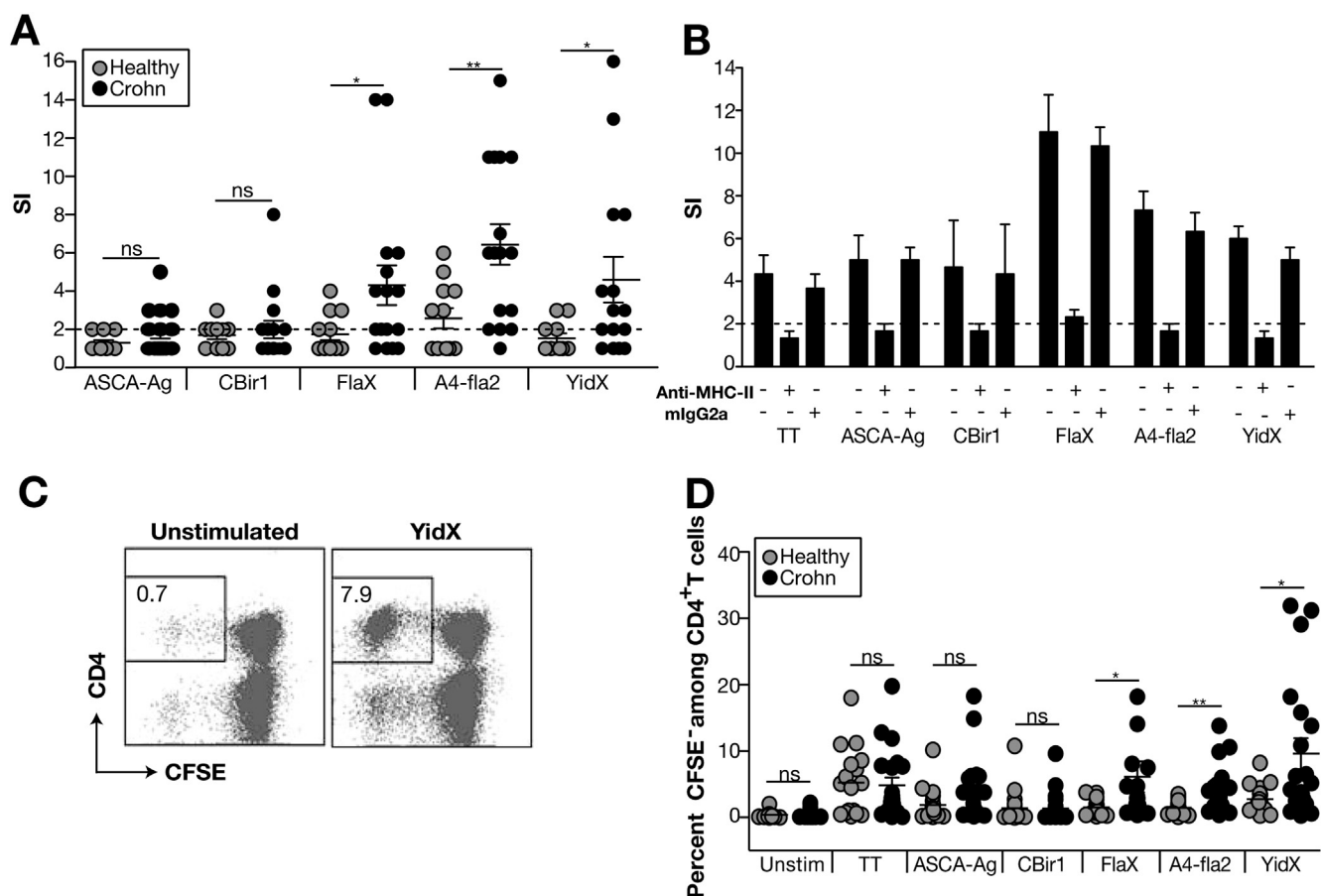


Figure 1. Enhanced proliferative response to commensal microbial antigens in CD patients. (A) Proliferation was measured by ³H-thymidine incorporation in PBMCs from healthy controls (n = 11) and CD patients (n = 16) cultured in the presence of microbial antigens for 7 days. Stimulation index (SI): counts per million from stimulated cells/counts per million unstimulated controls. A dashed line was set at a stimulation index of 2 and indicates the threshold for antigen-specific proliferation. (B) Proliferation measured by ³H-thymidine incorporation of PBMCs from CD patients (n = 3) cultured with microbial antigens in the presence of a human anti-major histocompatibility complex (MHC)-II antibody (anti-HLA-DP-DR-DQ) or isotype control (mIgG2a) for 7 days. (C) Representative fluorescence-activated cell sorting dot plots of CFSE dilution of CD4⁺ T cells after 7 days of culture with YidX. Numbers indicate the frequency of CFSE⁻ cells among live CD4⁺ T cells. (D) Percentage of CFSE⁻ CD4⁺ T cells in CFSE-labeled PBMCs from healthy controls (n = 18) and CD patients (n = 21). Means ± SEM. ns > .05, *P ≤ .05, **P < .01.

supernatant was determined by enzyme-linked immunosorbent assay following the manufacturer's instructions (R&D Systems).

Statistical Analysis

For 2-group comparisons, the 2-tailed Mann-Whitney-Wilcoxon test was used. One-way analysis of variance was performed using the Kruskal-Wallis statistics test or the Friedman test, both followed by the Dunn post-test. Data associations were analyzed using a Spearman rank correlation test. Statistical analyses were performed using Prism4 (Graphpad Software, San Diego, CA). Error bars show the mean and SEM. P values of .05 or less were considered statistically significant.

Results

Enhanced CD4⁺ T-Cell Proliferation to Commensal Microbial Antigens in CD Patients

We first measured PBMC proliferation to ASCA-Ag, CBir1, FlaX, A4-fla2, and YidX. These antigens were selected based

on reported increased sera reactivity in CD.^{7,9,10,27} CD patients (n = 16) showed a significantly higher stimulation index (defined as the fold-change in proliferation compared with unstimulated PBMCs) than healthy controls (n = 11) in response to FlaX, A4-fla2, and YidX stimulation (Figure 1A). The detected cell proliferation was attributable to antigen-specific-CD4⁺ T cells because it was abrogated specifically by an anti-major histocompatibility complex-II-blocking antibody (Figure 1B). To identify and further characterize antigen-responding CD4⁺ T cells, we used a CFSE-based dilution assay to quantify the percentage of CD4⁺ T cells that proliferated upon antigen stimulation (Figure 1C). TT was included in this assay as a non-commensal-derived bacterial antigen. An independent cohort of healthy controls (n = 18) and CD patients (n = 21) was used for this measurement; of those, 6 CD patients overlapped with data in Figure 1A. Similar to the total PBMC proliferation measured by thymidine incorporation, the percentage of proliferating (CFSE⁻) CD4⁺ cells was significantly higher in CD patients

compared with healthy controls upon FlaX, A4-fla2, and YidX, but not ASCA-Ag or CBir1 stimulation (Figure 1D). In contrast, the proliferative CD4⁺ T cell response to TT was comparable in CD patients and controls, strongly suggesting that increased reactivity to microbial components is a characteristic feature of CD patients.

We focused on FlaX, A4-fla2, and YidX because they drive higher PBMC proliferation (Figure 1A and D) in CD patients and analyzed the co-occurrence of antigen-specific T-cell reactivity. According to data shown in Figure 1A, 75% of CD patients (12 of 16) reacted to at least 1 of the 3 bacterial proteins (stimulation index, ≥ 3); of those, 83% (10 of 12) responded to more than one antigen (Supplementary Figure 1A). Regarding patients included in Figure 1D, more than 60% (14 of 21) showed CD4⁺ T cell reactivity to at least 1 of the 3 bacterial proteins. Of those, 78% (11 of 14) responded to more than 1 bacterial protein (Supplementary Figure 1B).

Next, we determined serologic responses to FlaX, A4-fla2, and YidX in CD patients (n = 19) and healthy controls (n = 17) for whom we had measured the antigen-specific CD4⁺ T-cell response (Figure 1D). Supplementary Figure 2A shows the comparative scatterplot of IgG serologic responses to FlaX, A4-fla2, and YidX in CD patients. Serologic positivity to each antigen was determined as described in the Supplementary Materials and Methods section.

More than 70% (14 of 19) presented with either a CD4⁺ T cell and/or an antibody response. Eighty-five percent of those (12 of 14) had combined serologic and CD4⁺ T-cell responses to at least 1 of the 3 antigens (Supplementary Figure 2B). Regarding each independent antigen, we found that 54% (6 of 11), 58% (7 of 12), and 58% (7 of 12) of the patients presented combined CD4⁺ T cell and antibody responses to FlaX, A4-fla2, and YidX, respectively (Supplementary Figure 2C).

Overall we show that CD patients have increased proliferative CD4⁺ T-cell responses to FlaX, A4-fla2, and YidX, and that these correlate in most individuals with the presence of specific IgG in serum.

We also analyzed the association of antigen-specific T cell and antibody responses with the patients' characteristics (included in Supplementary Table 1), and treatment at the time of the study. Although we did not find any significant association between the T-cell responses and any of the patient variables (data not shown), we did observe a positive association between the presence of YidX-specific antibodies and disease location because a higher number of patients with a positive serologic response present with ileocolonic (L3) disease (Supplementary Figure 3A). Interestingly, the presence of YidX-specific antibody responses also correlated positively with longer disease duration (Supplementary Figure 3B).

FlaX-, A4-fla2-, and YidX-Specific CD4⁺ T Cells From CD Patients Show a Th1 and Th17 Proinflammatory Phenotype

To define the cytokine profile of microbial antigen-driven T-cell responses, we first measured interferon

(IFN)- γ and IL17 in culture supernatants of total PBMCs stimulated for 7 days with TT, ASCA-Ag, CBir1, FlaX, A4-fla2, and YidX. We looked at IFN- γ and IL17 because they are characteristic of Th1 and Th17 responses, respectively, both of which have been associated with gut inflammation in CD.²⁸ Both cytokines were increased significantly in CD compared with control supernatants in response to FlaX, A4-fla2, and YidX (Supplementary Figure 4A and B). IFN- γ also was overproduced significantly by CD patients in response to ASCA-Ag, however, there was no difference in IL17 secretion. No differences in cytokines secretion were detected in response to CBir1. Interestingly, neither IFN- γ nor IL17 secretion induced by stimulation of PBMCs with the noncommensal antigen TT were significantly different in CD compared with healthy controls.

To specifically measure cytokine production by antigen-specific CD4⁺ T cells, we performed intracellular IFN- γ and IL17 staining on CFSE-labeled PBMCs cultured with TT, FlaX, A4-fla2, or YidX (Figure 2A) in an independent group of CD patients and controls. We observed that TT-specific CD4⁺ T cells mainly produced IFN- γ (Figure 2B), both in healthy individuals and CD patients. In contrast, FlaX-, A4-fla2-, and YidX-specific CD4⁺ T cells presented a mixed IFN- γ and IL17 profile. Although the percentage of single IFN- γ ⁺ IL17⁻ cells (Th1) was similar for CD patients and controls (Figure 2B), the frequency of IL17-producing T cells was significantly higher in the former. Remarkably, we noted significantly higher frequencies of IL17⁺ IFN- γ ⁻ single- (Th17) and IL17⁺ IFN- γ ⁺ double-positive (Th17/Th1) CD4⁺ T cells that recognized FlaX, A4-fla2, and YidX in CD patients compared with control individuals (Figure 2C and D). These data indicate that commensal-specific CD4⁺ T cells in CD patients present a Th17 and Th17/Th1 phenotype upon antigen recall that was not observed in T cells responding to the same antigen in healthy controls.

Commensal Antigen-Specific CD4⁺ T Cells From CD Patients Present a Th17-Related Transcriptional Signature

Next, we stimulated CFSE-labeled PBMCs with the commensal antigens FlaX, A4-fla2 or YidX, and sorted out CD4⁺ CFSE⁻ (proliferating) cells from a different cohort of CD patients and healthy controls. Freshly sorted cells then were restimulated with their cognate antigen, and autologous irradiated PBMCs were used as antigen-presenting cells. Total RNA from expanded antigen-specific T cells then was isolated. The whole genomic transcriptional signature of a pool of FlaX-, A4-fla2-, and YidX-specific CD4⁺ T cells was interrogated by microarray analysis and compared CD individuals (n = 10) with healthy controls (n = 8). Differential expression analysis of the microarray identified 299 genes whose expression was significantly different in CD patients compared with controls ($P \leq .05$; $|FC| \geq 1.5$) (Figure 3 and Supplementary Table 2). Among these genes, 37 belonged to the Th17 and Th17.1 transcriptional signatures (marked in purple in Figure 3), as described by Ramesh et al.²⁹ This included the up-regulation of *CCR6*, *IL17F*, *RORC*, *CCL20*, and *IL26* in CD

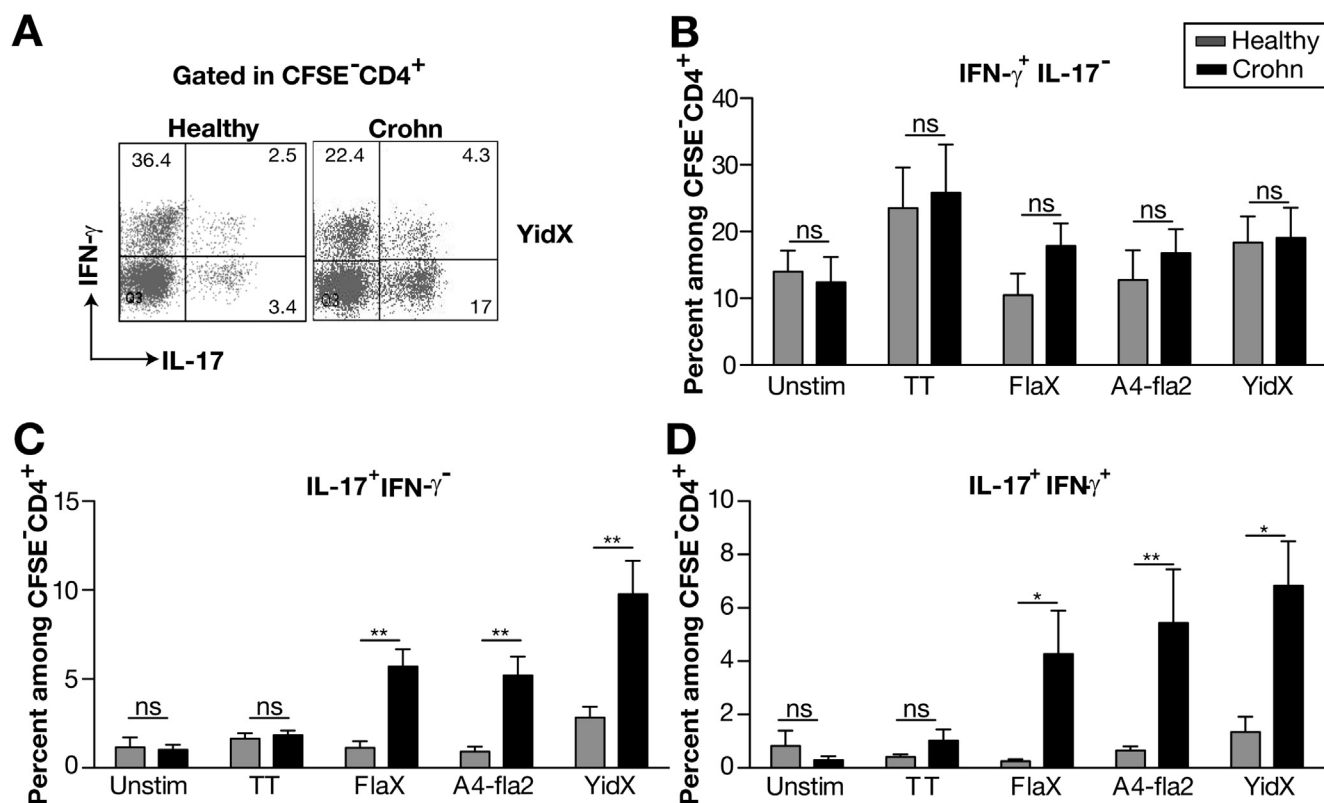


Figure 2. FlaX-, A4-fla2-, and YidX-specific CD4⁺ T cells from CD patients show a Th17/Th1 proinflammatory phenotype. CFSE-labeled PBMCs from healthy controls ($n = 8$) and CD patients ($n = 9$) were cultured alone or in the presence of TT, FlaX, A4-fla2, or YidX antigen for 7 days. (A) Representative fluorescence-activated cell sorting plots for IFN- γ and IL17 staining gated on live CFSE⁺ CD4⁺ T cells stimulated with YidX. (B) Frequency of IFN- γ ⁺ IL17⁻, (C) IL17⁺ IFN- γ ⁻, (D) IL17⁺ IFN- γ ⁺ among live CFSE⁺ CD4⁺ T cells. Means \pm SEM. ns $> .05$, * $P \leq .05$, ** $P < .01$. MHC, major histocompatibility complex; SI, stimulation index.

commensal antigen-specific CD4⁺ T cells, whereas other genes such as *PTGER2* were down-regulated. To validate the microarray data, a number of Th1 and Th17 signature genes were identified by real-time polymerase chain reaction in an independent group of samples from healthy controls ($n = 5$) and CD patients ($n = 6$). Real-time polymerase chain reaction analysis showed higher expression of *RORC*, *IL17A*, and *IL17F* in FlaX-, A4-fla2-, and YidX-specific T cells from CD patients compared with healthy controls (Figure 4A–C). Also in agreement with microarray data, expression of *CCR6* and *PTGER2* was significantly up- and down-regulated, respectively, in A4-fla2- and YidX-specific T cells from CD patients, but not in FlaX-specific T cells (Figure 4D and E). In addition, the expression of *CCL20* was higher in A4-fla2- and YidX-specific T cells from CD patients compared with controls (Figure 4F). Importantly, the Th1 signature genes *TBX21* and *IFNG*, and tumor necrosis factor (*TNF*) α , were expressed similarly in healthy controls and CD microbial antigen-specific T cells (Figure 5).

To investigate the clonality of antigen-specific T cells, we analyzed the T-cell receptor (TCR) repertoire of TT-, FlaX-, A4-fla2-, and YidX-specific CD4⁺ T cells by measuring the complementarity determining region 3 distribution length using spectratyping. We observed a diverse TCR repertoire for the 3 commensal-specific T cells in all individuals with

few monoclonal expansions within the TCR β -variable region. However, we did not detect a bias in TCR β -variable use, excluding the possibility of a superantigen-driven expansion (Supplementary Figure 5).

Activated Commensal Antigen-Specific CD4⁺ T Cells From CD Patients Promote Epithelial Inflammatory Responses

To further characterize commensal-specific T cells from CD patients, we analyzed the expression of the intestinal-homing integrin $\beta 7$ on antigen-specific CD4⁺ T cells in a total of 10 patients. We observed significantly higher expression of $\beta 7$ in FlaX-, A4-fla2-, and YidX-specific T cells compared with TT-specific T cells from CD patients (Supplementary Figure 6). Given their characteristic Th17-biased phenotype, as well as their relative increase in $\beta 7$ surface expression, we asked whether these cells could drive inflammation in the intestinal mucosa. To test the effect of antigen-specific T cells from CD patients on healthy intestinal epithelium, we isolated whole intestinal crypts from non-IBD surgical specimens (Figure 6A) and cultured them in the presence of supernatants from activated sorted TT-, FlaX-, A4-fla2-, and YidX-specific CD4⁺ T cells obtained from an independent cohort of CD patients ($n = 5$).

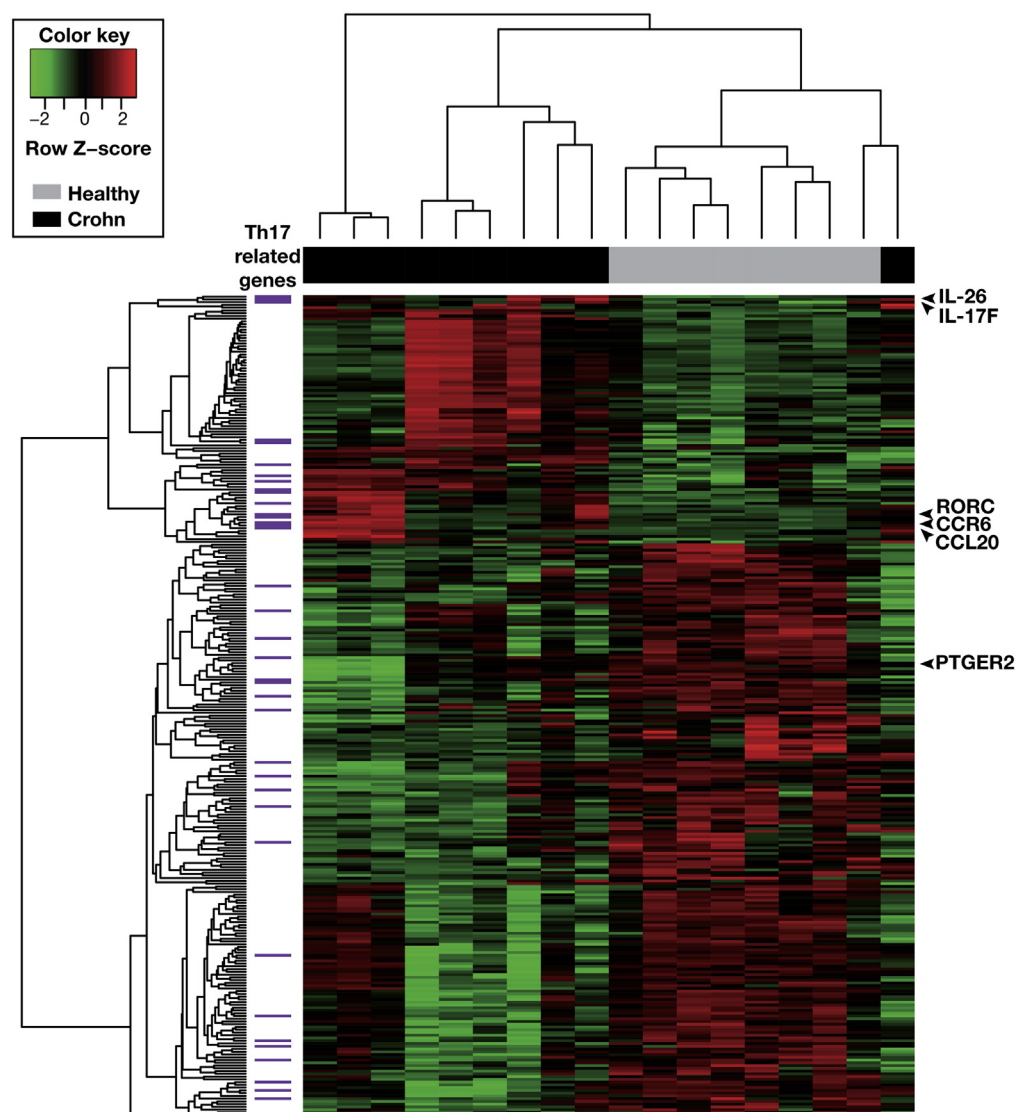


Figure 3. FlaX-, A4-fla2-, and YidX-specific CD4⁺ T cells from CD patients present Th17-biased transcriptional profiles. Differentially expressed genes by commensal microbial antigen (FlaX, A4-fla2, and YidX)-specific CD4⁺ T cells of healthy controls (n = 8) and Crohn's disease patients (n = 10) based on microarray analysis ($P \leq .05$ and $|\text{fold-change}| \geq 2$). Each row shows 1 individual probe (299 differentially expressed genes) and each column shows an experimental sample. Genes related to the Th17 and Th17.1 transcriptional signatures are marked in purple. An unsupervised hierarchical cluster method using Pearson distance and average linkage method was applied for gene and sample classification.

Treatment of isolated intestinal crypts with supernatants from anti-CD3/anti-CD28-activated commensal antigen-specific CD4⁺ T cells induced higher transcriptional expression (Figure 6B and 6D) and protein secretion (Supplementary Figure 7A and B) of the neutrophil-recruiting chemokines *CXCL8* and *CXCL1* compared with crypts that had been cultured with medium alone. Of note, crypts treated with supernatants from activated TT-specific CD4⁺ T cells did not up-regulate *CXCL1* or *CXCL8* expression. Supernatants of commensal-specific T cells also induced higher expression of *CCL20*, a chemoattractant for CCR6-expressing Th17 cells, on non-IBD intestinal crypts (Figure 6C) compared with those treated with supernatants derived from TT-specific T cells; nonetheless, the differences compared with nonactivated crypts were not significant. In contrast, we observed that supernatants from TT induced higher transcriptional and protein expression of the Th1-attracting chemokine *CXCL10* compared with unstimulated crypts (Figure 6E and Supplementary Figure 7C). These results suggest that commensal-specific T cells from

CD patients prompt a predominant neutrophil and Th17 recruitment to the intestinal epithelium.

IL17 Regulates Proinflammatory Chemokine Expression by Intestinal Epithelial Crypts

We next determined which cytokines were present in the T-cell supernatants used to stimulate intestinal epithelial crypts. As predicted by their transcriptional signatures, FlaX-, A4-fla2-, and YidX-specific T-cell supernatants contained high amounts of IL17A, IL17F, IL22, and CCL20 (Supplementary Figure 8A–D), whereas TT-specific T-cell supernatants contained very low concentrations of these cytokines. In contrast, we measured similar amounts of IFN- γ (Supplementary Figure 8E) and TNF- α (Supplementary Figure 8F) in all supernatants regardless of antigen specificity.

It has been observed that IL17 and TNF- α can act synergistically to drive *CXCL8*, *CXCL1*, and *CCL20* expression on epithelial cell lines, whereas IL17 represses the expression

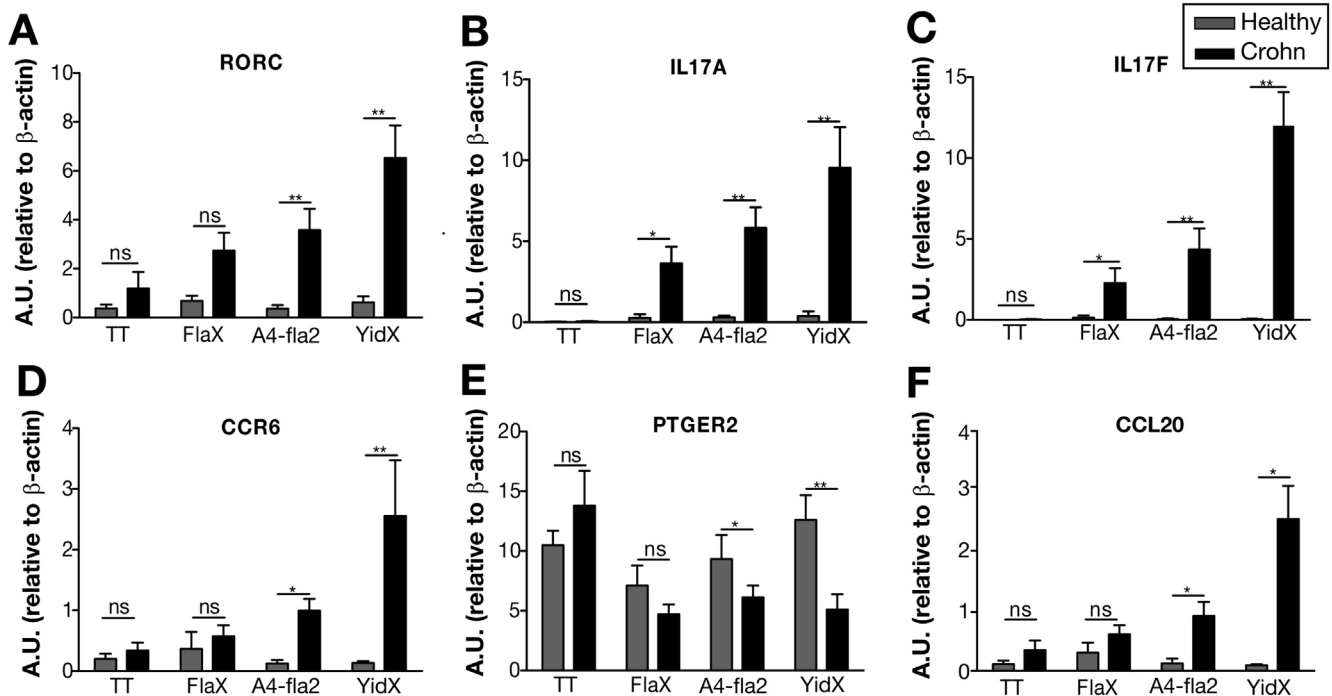


Figure 4. Differential expression of Th17-related genes by commensal antigen-specific CD4⁺ T cells from CD patients. Messenger RNA expression of (A) *RORC*, (B) *IL17A*, (C) *IL17F*, (D) *CCR6*, (E) *PTGER2*, and (F) *CCL20* genes assessed by real-time polymerase chain reaction in sorted antigen-specific CFSE⁻ CD4⁺ T cells from healthy controls (n = 5) and CD patients (n = 6). Arbitrary units (AU) relative to β -actin expression. Means \pm SEM. ns > .05, * $P \leq .05$, ** $P < .01$.

of *CXCL10*, even in the presence of TNF- α .³⁰ We observed a similar effect on primary intestinal epithelial crypts upon the addition of recombinant IL17 (r-IL17) and TNF- α because they induced increased *CXCL8*, *CXCL1*, and *CCL20* transcriptional expression (Supplementary Figure 9A–C). In contrast, *CXCL10* expression was inhibited when r-IL17 was added to the culture (Supplementary Figure 9D). Interestingly, neutralization of IL17 reduced the synergistic effect of r-IL17 and recombinant TNF- α in the induction of *CXCL8*, *CXCL1*, and *CCL20* (Supplementary Figure 9A–C), but it restored *CXCL10* expression (Supplementary Figure 9D). In line with this, the effect of FlaX-, A4-fla2-, and YidX-specific T-cell supernatants on *CXCL8*, *CXCL1*, and *CCL20* expression

in intestinal crypts was reduced upon neutralization of IL17 (Figure 7A–C). It is worth noting that neutralization of IL17 in commensal-specific T-cell supernatants induced higher expression of *CXCL10* on intestinal crypts (Figure 7D).

Discussion

The loss of tolerance toward commensal bacteria as a mechanism driving CD is widely accepted. To date, it is mostly understood as increased seroreactivity toward a variety of microbial antigens in CD.^{12,13,31–33} This suggests the existence of helper T cells reacting to the same commensal microorganisms. Despite the fact that CD4⁺ T

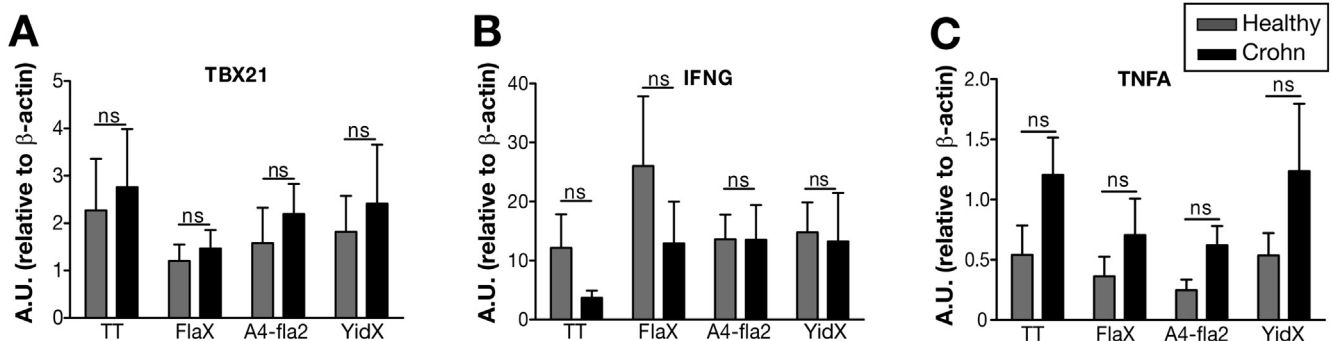


Figure 5. Th1-related genes and *TNFA* are not expressed differently by commensal antigen-specific CD4⁺ T cells from CD and healthy controls. Messenger RNA expression of (A) *TBX21*, (B) *IFNG*, and (C) *TNFA* genes assessed by real-time polymerase chain reaction in sorted antigen-specific CFSE⁻ CD4⁺ T cells from healthy controls (n = 5) and CD patients (n = 6). Arbitrary units (AU) relative to β -actin expression. Means \pm SEM. ns > .05.

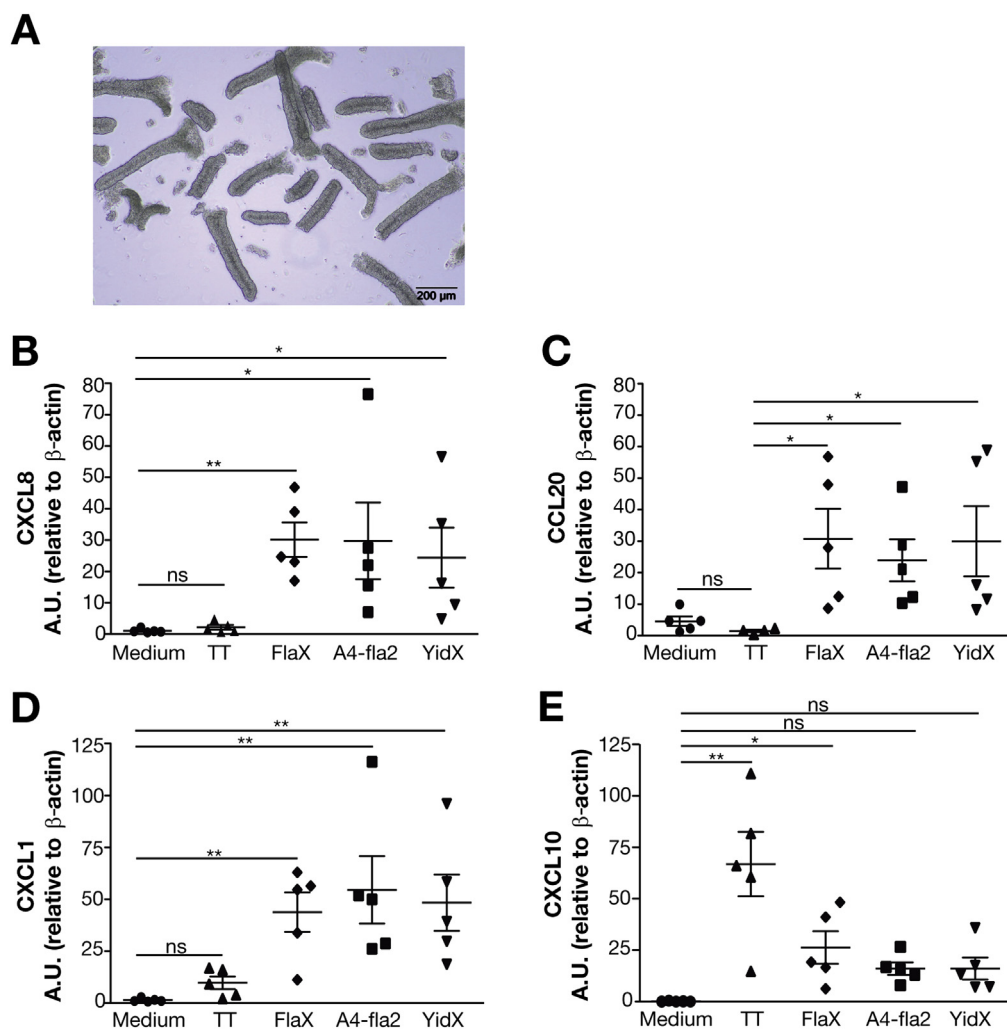


Figure 6. Activated FlaX-, A4-fla2-, and YidX-specific CD4⁺ T cells from CD patients promote intestinal inflammation. (A) Representative picture of whole intestinal crypts from non-IBD surgical specimens after 18 hours of culture. Relative gene messenger RNA expression of (B) CXCL8, (C) CCL20, (D) CXCL1, and (E) CXCL10 in intestinal crypts cultured with supernatants from activated antigen-specific CD4⁺ T cells from CD patients (n = 5). Means ± SEM. ns > .05, *P ≤ .05, **P < .01.

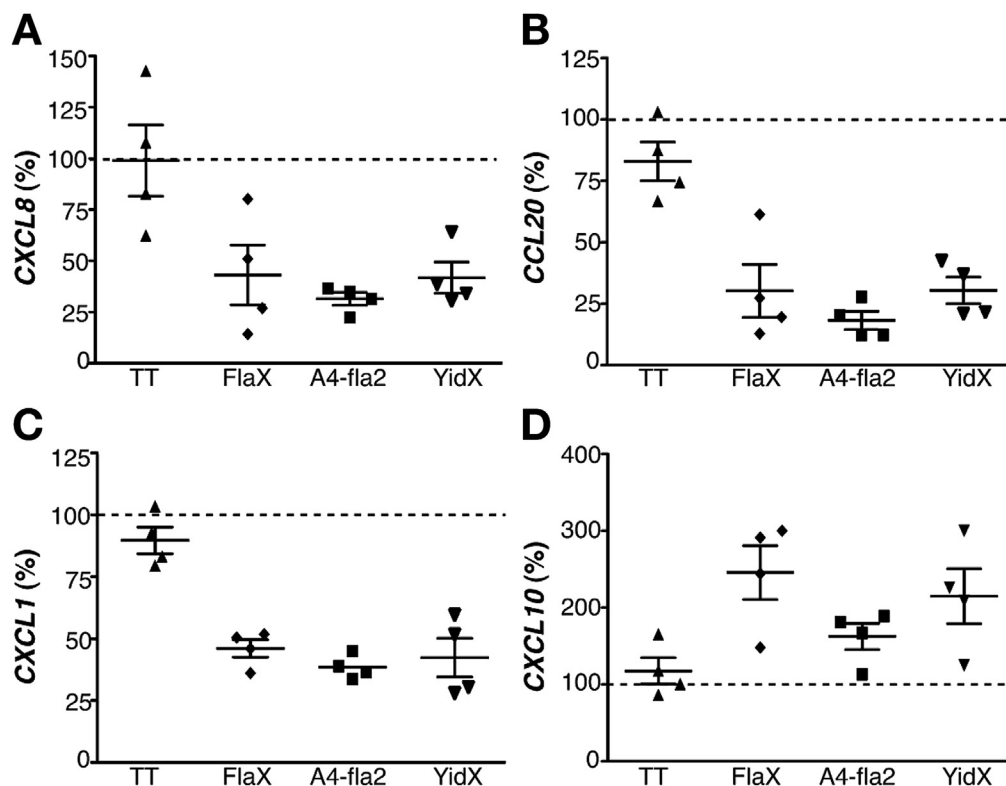
cells are regarded as key players in CD pathogenesis, there still are limited data on the antigen specificity of the T-cell response in CD.^{16,23,25,34,35}

Here, we provide novel evidence of the existence of commensal microbial-specific CD4⁺ T cells in the peripheral blood of both CD patients and healthy individuals. Importantly, proliferation in response to commensal antigen stimulation was enhanced in CD patients compared with healthy controls. The fact that healthy individuals present with circulating commensal-specific CD4⁺ T cells is not completely unexpected. In fact, a T-cell responses to *E coli* proteins have been observed previously in healthy subjects.³⁵ In other immune-mediated diseases, such as multiple sclerosis, T-cell reactivity to self-antigens (myelin) has been reported in healthy controls despite the fact that myelin-specific T cells showed increased activation in multiple sclerosis patients^{36,37} and a different functional inflammatory profile.³⁸ Moreover, it has been described recently that healthy individuals present IgG antibodies to gut microbial antigens in serum, suggesting that microbial-specific CD4⁺ T-cell helper cells can similarly develop in the absence of CD.³⁹

A key finding of our study was that FlaX-, A4-fla2-, and YidX-driven CD4⁺ T-cell responses in CD patients showed a distinct inflammatory phenotype, a response characterized by secretion of high amounts of TNF-α, IFN-γ, IL17A, IL17F, and CCL20. These differences were confirmed by transcriptional analysis of sorted FlaX, A4-fla2, and YidX-specific CD4⁺ T cells. Through this analysis we identified significant differences in the expression of *RORC*, *IL17A*, *IL17F*, *CCR6*, *CCL20*, and *PTGER2* in commensal-specific T cells from CD patients compared with control subjects. In contrast, we observed no difference in Th1-related genes and *TNFA* between these 2 groups.

Importantly, the percentage of Th17 and Th17/Th1 cells among commensal-specific CD4⁺ T cells in CD patients was remarkably higher than in healthy controls. In fact, Th17/Th1 cells have been found in CD at unusually higher rates, together with conventional Th1 and Th17 cells.^{28,40} It also has been postulated that pathogenic Th17 cells can be found within the Th17/Th1 (Th17.1) subset in CD,²⁹ suggesting that FlaX-, A4-fla2-, and YidX-specific T cells play a proinflammatory role in CD. Data in experimental models show that Th17 cells can give rise in vivo to Th1 cells and that this

Figure 7. IL17 neutralization reduces the expression of neutrophil and Th17-recruiting chemokines by intestinal epithelial crypts. Chemokine levels of (A) *CXCL8*, (B) *CCL20*, (C) *CXCL1*, and (D) *CXCL10*, on intestinal crypts after 18 hours of culture with supernatants from activated antigen-specific CD4⁺ T cells from CD patients pretreated with anti-IL17 monoclonal antibody ($n = 4$). Chemokine levels after stimulation with untreated supernatants were set as the 100% value for each donor (dashed line), and expression for stimulation with anti-IL17-treated supernatants is shown as the percentage thereof. Means \pm SEM.



ability to produce IFN γ is required to induce colitis.⁴¹ Remarkably, this study also showed that Th17 also supports the de novo generation of pathogenic Th1 cells.

To provide additional specificity to our observations, we included throughout our study a noncommensal bacterial protein (TT) for which all appropriately vaccinated individuals presented memory TT-specific CD4⁺ T cells in peripheral blood. We show that the CD4⁺ T-cell proliferative response to the disease-unrelated protein TT is comparable in CD patients and healthy controls. Moreover, TT elicits a predominantly Th1 response both in CD and in healthy controls. Collectively, this would show that CD4⁺ T cells from CD patients are not generally biased toward a highly proliferative Th17 phenotype, but rather that this response is geared specifically toward defined gut commensal antigens. The marked differences among commensal-specific T cells from healthy controls and CD patients suggest that despite reacting to the same protein antigens, their priming may have taken place in strikingly different environments. This notion is supported further by the fact that TT-specific T cells, which have been primed in the presence of the same vaccine adjuvant (alum) in both healthy controls and CD patients, do not show functional differences among patients and controls. In addition, it has been shown that during gastrointestinal infection with *Toxoplasma gondii*, commensal-specific T cells (CBir1 transgenic T cells) differentiated into Th1 cells, while giving rise to Th17 cells upon chemical disruption of the epithelial barrier in mice. These data thus indicate that microbiota-specific T cells are shaped by signals provided by the inflammatory milieu rather than by antigen specificity.⁴²

Interestingly, a recent genetic study in IBD patients identified a causal association between the HLA-II allele HLA-DRB1*01:03 and CD,⁴³ indicating that the adaptive immune response plays a central role in CD. This study supports our findings and the notion that antigen-specific CD4⁺ T cells could contribute to intestinal inflammation in the context of CD.

It is likely that these circulating commensal-specific T cells in CD patients are recruited to the intestinal mucosa during disease flares. In fact, we show that commensal-specific T cells present an overall higher $\beta 7$ expression profile compared with TT-specific cells in CD patients. High $\beta 7$ (which in peripheral blood is associated primarily with $\alpha 4$) favors binding to the endothelial adhesion molecule mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1), which is expressed constitutively by high endothelial venules and the inflamed intestinal vascular endothelium. To test the proinflammatory potential of commensal-specific T cells from CD patients in the intestine, we cultured non-IBD primary epithelial crypts with T-cell-derived supernatants. Importantly, we found that the cytokine cocktail produced by commensal-specific T cells, but not by noncommensal-specific T cells (TT), in CD patients elicited a proinflammatory response in intestinal epithelial crypts (higher expression of *CXCL1*, *CXCL8*, and *CCL20*). Thus, this effect may be promoted by the synergy between IL17A and other secreted cytokines such as TNF- α (all highly secreted by commensal-specific CD T cells). This would be in agreement with our observation regarding primary intestinal epithelial cells and other studies involving recombinant IL17 and TNF- α on intestinal

epithelial cell lines.³⁰ Hence, we believe that in CD patients, commensal-specific T cells, which show a mixed Th17/Th1 phenotype, may exacerbate intestinal inflammation by creating a feedback loop that favors the accumulation of Th17 cells (CCL20) and neutrophils (CXCL1 and CXCL8) at the site of inflammation.

An important issue we addressed was whether CD4⁺ T-cell responses to microbial antigens occurred simultaneously with the presence of specific antibodies in serum. We observed that, in most patients, CD4⁺ T-cell responses to FlaX, A4-fla2, or YidX were accompanied by serologic responses to the antigens studied. Although the number of patients may be too low to make any definitive conclusions, our results suggest that detection of specific antibodies to these antigens can act as a reliable surrogate marker for the presence of the cognate T-helper response in CD patients. Remarkably, anti-YidX antibodies in serum independently correlated with ileocolonic disease location and with long-term disease despite the small number of patients analyzed. Previous studies using larger cohorts of CD patients identified correlations between antibodies to other microbial proteins with complicated disease behavior, including small-bowel disease location.^{3,8}

Although our study does not provide data on the microbial-specific response in ulcerative colitis (UC), it has been described previously that YidX-specific serologic responses are more abundant in UC patients, in relation to other microbial components, compared with CD patients.¹⁰ However, antibody responses to FlaX and A4-fla2 arise in only a small percentage (6%) of UC patients compared with CD patients (57% and 59%, respectively).⁸ Because increasing evidence has indicated that Th17 cells may be important contributors to intestinal inflammation in UC,¹⁹ it would be interesting to investigate the antigen specificity of T cells in patients with UC, in particular to YidX.

Overall, our data would point to IL17 as a desirable target in treating CD. Nonetheless, recent clinical data unexpectedly showed the negative effects of such therapy in 2 phase II studies,^{44,45} despite having shown efficacy in other immune-mediated diseases.⁴⁶ The results of these clinical trials highlighted the important role of IL17 in controlling microbial and fungal growth in subjects with ongoing mucosal ulceration because its blockade induced severe infections, including mucocutaneous candidiasis in treated patients. Furthermore, IL17 also is produced by other immune cells in the intestine, such as innate-like lymphocytes, natural killer cells, natural killer-T cells, and T-regulatory cells, which may play an important role in the regulation of intestinal homeostasis.^{47,48} The identification and characterization of commensal-specific CD4⁺ T cells offers the opportunity to specifically target potential pathogenic CD4⁺ T cells without altering other IL17-producing immune cells that may be beneficial to control fungal growth and intestinal homeostasis. Targeting FlaX-, A4-fla2-, and YidX-specific CD4⁺ T cells, as opposed to blocking IL17 alone, may offer a valuable therapeutic option because it may impact other cytokines secreted by these cells, such as TNF- α , which appears to act synergistically on intestinal inflammation in CD.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at <http://dx.doi.org/10.1053/j.gastro.2016.05.050>.

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Acknowledgments

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Conflicts of interest

This author discloses the following: Sharat Singh was employed by Prometheus Laboratories. The remaining authors disclose no conflicts.

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Supplementary Materials and Methods

Antigen Stimulation of PBMCs

PBMCs were isolated from heparinized peripheral blood of healthy controls or patients with CD by Ficoll (Sigma-Aldrich) gradient centrifugation. Cells were cultured in X-VIVO 15 medium (Bio Whittaker, Lonza, Belgium) supplemented with 2% inactivated AB human serum (Sigma-Aldrich) for 7 days. For T-cell proliferation screening, 1×10^5 PBMCs were cultured with the microbial commensal antigens ASCA-Ag, CBir1, FlaX, A4-fla2, and YidX (Prometheus Laboratories, Inc) at 1 μ g/mL for 7 days. Human r-IL2 (20 IU/mL; eBioscience) was added on day 3 of culture. On day 6, tritiated thymidine (1 μ Ci/well; Amersham) was added to the culture in triplicate. The ³H thymidine incorporation took place during the last 16 hours of culture. For major histocompatibility complex-II blocking, anti-HLA-DR, -DP, -DQ (BD Pharmingen) was added at 20 μ g/mL to the culture. For CFSE (Cell Trace CFSE cell proliferation kit; Life Technologies) staining, freshly isolated PBMCs were incubated with CFSE at 5 mmol/L according to the supplier's instructions. Cells then were plated at 1×10^6 cells/mL and cultured with TT (Sigma-Aldrich) and microbial commensal antigens (Prometheus Laboratories, Inc), all at 2 μ g/mL for 7 days.

Enzyme-Linked Immunosorbent Assays of Serum Antimicrobial Antibodies

Serum antibodies to FlaX, A4-fla2, and YidX were measured by enzyme-linked immunosorbent assay in 37 samples (18 healthy individuals and 19 CD patients). Briefly, enzyme-linked immunosorbent assay plates were coated at 4°C overnight with 4 μ g/well of FlaX, A4-fla2, YidX, or with an irrelevant protein for nonspecific background subtraction. Plates were blocked with 3% bovine serum albumin in phosphate-buffered saline for 1.5 hours at room temperature. After washing, serum was added at 1:100 dilution in 0.1% bovine serum albumin-phosphate-buffered saline for 2 hours at room temperature. Afterward, plates were washed and incubated for 1 hour with a 1:50,000 dilution of a horseradish-peroxidase-conjugated goat anti-human γ chain specific antibody (Jackson ImmunoResearch Labs, West Grove, PA). After washing, plates were incubated with tetramethylbenzidine substrate (eBioscience). Absorbance was read at 620 nm on a microplate reader (Molecular Devices, Sunnyvale, CA). Specific optical density was calculated for each sample and antigen after subtracting its background optical density.

Seroreactivity to each antigen was determined as an optical density value greater than the mean optical density \pm standard deviation in a group of healthy controls.

Flow Cytometry

For intracellular cytokine staining, cells were restimulated with phorbol myristate acetate and ionomycin in the presence of brefeldin A (all from Sigma-Aldrich) at

25 ng/mL, 0.5 μ g/mL, and 10 μ g/mL, respectively, for the final 4 hours of culture. Cells were fixed and permeabilized with FIX and PERM (Caltag, Life Technologies) according to the manufacturer's instructions. Cells were stained with a LIVE/DEAD fixable violet dead cell stain kit (Life Technologies), anti-CD4 (RPA-T4; BD Biosciences), anti-IL17 (clone 64DE17; eBioscience), and anti-IFN- γ (clone 4S.B3; eBioscience) conjugated with different fluorochromes.

To assess the expression of integrin β 7 on viable CFSE⁺ CD4⁺ cells, the antibody anti-integrin β 7 (clone FIB504; BD Biosciences) was used.

Stained cells were acquired in a Fluorescence-Activated Cell Sorting Canto II (BD Bioscience) and analyzed with BD Biosciences Fluorescence-Activated Cell Sorting Diva Software v6.1.1.

Soluble Cytokine and Chemokine Analysis

For IL17 detection capture and detection, antibodies were obtained from eBioscience. For IFN- γ an enzyme-linked immunosorbent assay kit (BD OptEIA, Human IFN- γ Enzyme-Linked Immunosorbent Assay Set, BD Biosciences) was used following the manufacturer's instructions. Simultaneous detection of IL17A, IL17F, IL22, CCL20, IFN- γ , and TNF- α in the supernatants of antigen-specific CD4⁺ T cells was performed using the multiplex cytokine assay MILLIPLEX Human Th17 magnetic bead (EMD Millipore Corporation).

For the detection of chemokines in crypt culture supernatants, enzyme-linked immunosorbent assay kits were used. For CXCL1 (Human CXCL1/GRO α DuoSet Enzyme-Linked Immunosorbent Assay), CXCL10 (Human CXCL10/IP-10 DuoSet Enzyme-Linked Immunosorbent Assay), and for CXCL8 (Human CXCL8/IL-8 DuoSet ELISA) enzyme-linked immunosorbent assay kits were used, all from R&D Systems. They were used according to the manufacturer's instructions.

Cell Sorting of Antigen-Specific CD4⁺ T Cells and RNA Isolation

CFSE-labeled PBMCs were cultured in the presence of TT, FlaX, A4-fla2, or YidX antigens at 2 μ g/mL. Recombinant IL2 (R&D systems) (20 IU/mL) was added to the culture on day 7. Cells were harvested at day 14, washed, and stained using a LIVE/DEAD fixable violet dead cell stain kit (Life Technologies) and anti-CD4⁺ (RPA-T4; BD Bioscience). Viable CFSE⁺ CD4⁺ cells were sorted in a Fluorescence-Activated Cell Sorting Aria II and cultured with autologous irradiated PBMCs pulsed with 2 μ g/mL of TT, FlaX, A4-fla2, or YidX. IL2 (10 IU/mL) was added on day 5. After 10–12 days of culture, T cells were harvested, washed in PBS, and resuspended in TRIzol (Ambion, Foster City, CA). RNA was isolated using RNeasy kit (Qiagen, Hilden, Germany) according to the supplier's instructions.

Intestinal Crypt Isolation and Culture

Non-IBD intestinal epithelial crypts were isolated from intestinal tissue as previously described.¹ For short-term

crypt culture, 30 isolated crypts/25 μ L Matrigel (BD Biosciences) were plated and cultured in either complete crypt culture medium: advanced Dulbecco's modified Eagle medium/F12 (Thermo Fisher Scientific), GlutaMax (Thermo Fisher Scientific), 10 mmol/L HEPES (Sigma), N-2 (1 \times) (Thermo Fisher Scientific), B-27 without retinoic acid (1 \times) (Thermo Fisher Scientific), 1 mmol/L N-acetyl-L-cysteine (Sigma), 500 ng/mL RSP01 (Sino Biologicals, Beijing, China), 100 ng/mL human Noggin (Peprotech, Rocky Hill, NJ), 500 nmol/L LY2157299 (Axon MedChem, Groningen, The Netherlands), normocin 100 μ g/mL and 1 mmol/L valproic acid (Sigma-Aldrich), or in medium containing supernatants from activated sorted antigen-specific CD4⁺ T cells (1:1). Antigen-specific T-cell supernatants were obtained from restimulating 1×10^5 cells with anti-CD3 (BD Biosciences) and anti-CD28 (BD Biosciences) at 1 μ g/mL for 5 days.

Neutralization of IL17 was achieved by preincubation of T-cell supernatants for 1 hour with monoclonal anti-IL17A (R&D Systems). After overnight culture at 37°C and 5% CO₂, Matrigel embedded crypts were resuspended in 500 μ L TRIzol (Ambion) and total RNA was isolated using the RNeasy Kit (Qiagen).

Microarray Data Analysis

The derived RNA from sorted antigen-specific CD4⁺ T cells was hybridized to a high-density oligonucleotide Affymetrix Human Genome U219 Array Plate (Affymetrix Santa Clara, CA). Raw data were analyzed using Bioconductor tools (version 2.13²) in R (version 3.1.0³) using the CG (guanine-cytosine) content-adjusted robust multi-array algorithm to normalize and linear models for microarray data for differential expression analysis. Microarray raw data (.cel files) and processed data are accessible through GEO series accession number GSE70469. The transcriptional signature of Th17 or Th17.1 was obtained from GSE49703.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA from sorted CD4⁺ T cells or stimulated whole crypts was transcribed to complementary DNA using reverse transcriptase (High Capacity cDNA RT kit, Applied Biosystems, Carlsbad, CA). Polymerase chain reaction was performed in TaqMan Universal Polymerase Chain Reaction Master Mix and probes (Applied Biosystems). Reverse transcription was performed in a 96-well thermocycler (Veriti 96W; Applied Biosystems). TaqMan real-time polymerase chain reaction was used to detect transcripts of *RORC*, *IL17A*, *IL17F*, *CCL20*, *CCR6*, *PTGER2*, *TBX21*, *IFNG*, *TNFA*, *CXCL1*, *CXCL8*, and *CXCL10*. Fluorescence was detected in an ABI PRISM 7500 Fast RT- Polymerase Chain Reaction System (Applied Biosystems). Primers and probes for each sequence were obtained as inventoried TaqMan gene-expression assays (Applied Biosystems). B-actin was used as a reference gene. Fluorescence was detected using an ABI PRISM

7500 Fast RT-Polymerase Chain Reaction System (Applied Biosystems).

Multiplex Polymerase Chain Reaction Amplification of β Chain Complementarity Determining Region 3

Total messenger RNA was extracted with an RNeasy Plus Mini Kit (Qiagen) and reverse-transcribed using oligo-deoxythymidine primer. As a control of mRNA integrity, the glyceraldehyde-3-phosphate dehydrogenase gene was amplified from all samples. Multiplex polymerase chain reaction was adapted from Chitnis and Pahwa.⁴ Amplification mixtures, including 24 Vb gene families, were performed in 15- μ L reactions containing 1.2 mL of the forward Vb primer MIX and 0.75 mL of the reverse Cb primer at 5 mmol/L, 10 \times each deoxynucleoside triphosphate (2.5 mmol/L), 10 \times polymerase chain reaction buffer with 2 mmol/L MgCl₂, 40 ng complementary DNA, and 0.6 U of Taq polymerase (Biotools, Madrid, Spain). Polymerase chain reaction amplification was 3 minutes at 97°C, 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute, and 10 minutes at 72°C. Primer mixes were as follows: MIX-A (TCR β -variable 9/30/13), MIX-B (TCR β -variable 20/29/12), MIX-C (TCR β -variable 28/6-5), MIX-D (TCR β -variable 5/25), MIX-G (TCR β -variable 3/14), and MIX-H (TCR β -variable 5-6/10), 2.5 mmol/L of each primer; MIX-E (TCR β -variable 7/15), MIX-F (TCR β -variable 4/11), MIX-I (TCR β -variable 6-2/24), and MIX-J (TCR β -variable 18/27), for which primers were at 1.7 and 3.3 mmol/L, respectively; and MIX-K (TCR β -variable 23) and MIX-L (TCR β -variable 2) at 2.5 mmol/L. For each multiplex polymerase chain reaction, a negative control was run in parallel to exclude possible contaminations.

Complementarity Determining Region 3 Length Analysis by Spectratyping

Run-off extension was performed using multiplex polymerase chain reaction products as a template and the reverse primer Cb 5' labeled with 6-carboxyfluorescein 6-FAM, consisting of 3 \times 2 minute cycles at 95°C, 2 minutes at 55°C, and 20 minutes at 72°C. Fluorescent polymerase chain reaction products were run with a size marker GS400HD (Applied Biosystems) and were length-separated by the ABI 3130XL analyzer (Applied Biosystems). Data were analyzed with the software PeakScanner (Applied Biosystems). Peak areas were quantified and normalized with the size marker to determine the total normalized area corresponding to each TCR β -variable family. To assess the presence of monoclonal T-cell expansions, the relative fluorescence intensity was calculated as follows: relative fluorescence intensity (%) = (peak area/total Vb area).^{5,6} A complementarity determining region 3 peak with a relative fluorescence intensity greater than 50% was considered a monoclonal expansion.

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