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ORIGINAL ARTICLE

IL26 modulates cytokine response and anti-TNF consumption in Crohn's disease patients with bacterial DNA

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Abstract
Interleukin IL26 supports killing of microbes and the innate sensing of bacterial-derived DNA (bactDNA). We evaluated the relationship between IL26 serum levels and bactDNA translocation in Crohn's disease (CD). We ran a prospective study on CD patients in remission. IL26 common polymorphisms, serum cytokines and complement protein, amplified-bactDNA, and anti-TNF-α were evaluated. In vitro PBMC analysis was performed. Three hundred and thirteen patients were included (mean CDAI: 83.6 ± 32.8; mean fecal calprotectin: 55.4 ± 35.3 μg/g). A total of 106 patients (33.8%) showed bactDNA and 223 patients (71%) had a var*IL26* genotype. BactDNA significantly correlated with increased IL26 levels compared with bactDNA-negative patients. PBMCs from var*IL26* patients significantly reduced *E. coli* killing capacity compared with wt*IL26*-genotyped patients. The stimulation with a recombinant IL26 protein reduced pro-inflammatory cytokines in response to *E. coli* in the var*IL26* cell supernatants. Serum anti-TNF-α levels in var*IL26* vs *wtIL26*-genotyped patients on biologics were significantly lower in the presence of bactDNA. Cells from var*IL26* vs wt*IL26*-genotyped patients cultured with *E. coli* DNA and infliximab showed a significant decrease in free anti-TNF-α concentration. A var*IL26* genotype was associated with the initiation of anti-TNF-α in CD patients during the 6-month follow-up. *IL26* polymorphisms may prevent bactDNA clearance and identify CD patients with a worse inflammatory evolution and response to therapy.

Key messages
- BactDNA translocation in CD is associated with an increased risk of relapse.
- IL26 is sensitive to bactDNA and modulates the inflammatory response in CD patients.
- The var*IL26* genotype is associated with reduced PMN capacity to kill bacteria.
- A var*IL26* genotype is associated with decreased levels of anti-TNF-α in CD patients.
- IL26 may help explain the role of bactDNA as a risk factor of flare in CD patients.

Keywords Crohn's disease · Bacterial translocation · Interleukin 26

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Introduction

The translocation of bacterial DNA (bactDNA) into the blood is a frequent and clinically relevant event in up to 40% of patients with Crohn's disease (CD) [1, 2], which is driven by an unbalanced interaction between the intestinal microbiota and the mucosal immune system in a genetically predisposed individual [3–6], leading to a sustained inflammatory milieu in these patients [7, 8]. The translocation of bactDNA has been associated with increased disease activity, and it has been identified as an independent risk factor of flare in the short term [9].

Interleukin 17-producing helper T (T_H) cells are involved in defense against fungi and extracellular bacterial infections, are increased in numbers in active CD patients [10], and produce cytokines associated with disease activity [11]. Interleukin IL26 is produced by T_H17 cells in colonic lessions of patients with active inflammatory bowel disease (IBD) [12]. IL26 is a 19 kDa member of the IL-20 cytokine family that signals through the IL-10R2-IL-20R1 heterodimeric receptor [13]. It facilitates killing of microbes, senses extracellular bactDNA, and promotes a potent proinflammatory response by inducing plasmocytoid dendritic cells (pDCs) to secrete interferon (IFN)-α [14]. The clearance of bactDNA might be compromised in the absence of IL26 availability and account for disease flares. Actually, the blockade of IL-17A with the human anti-IL-17A monoclonal antibody secukinumab leads to increased disease activity in CD patients [15].

Genome-wide association studies have identified single-nucleotide polymorphisms (SNPs) in the *IL26* gene that were associated with IBD [16, 17] and other inflammatory disorders, such as rheumatoid arthritis [18]. As IL26 promotes bacterial killing [14], we hypothesize that CD patients with a variant (var) *IL26* genotype may not adequately clear bactDNA fragments, facilitating upheld levels of proinflammatory mediators such as TNF-α. This might contribute to the significantly reduced levels of anti-TNF-α observed in patients with bactDNA compared to patients without bactDNA, considered as an indirect evidence of increased drug consumption in these patients [8], and would support the role of bactDNA translocation as a risk factor for relapse in CD patients [9].

In the present study, we have investigated the role of IL26 on the inflammatory and anti-TNF-α levels in response to bactDNA translocation in a large series of CD patients in remission.

Material and methods

Patients and study design

In this prospective, observational study, CD patients in remission, diagnosed and followed at Hospital General Universitario de Alicante, Spain, were consecutively included. Remission was defined as a CDAI < 150 and absence of clinical symptoms of relapse. Only patients, who did not change their established therapy in the previous month, were included. The diagnosis of CD was established according to standard clinical, endoscopic, histological, and radiographical criteria [19]. Patients treated with antibiotics in the previous 4 weeks, patients with signs of active infection, and those who refused to sign informed consent to participate in the study were excluded. The incidence of *IL26* gene polymorphisms in the control population was obtained from The 1000 Genomes Project Consortium [20] (www.1000genomes.org). The Ethics Committee of Hospital General Universitario de Alicante approved the study protocol.

Usual clinical and analytical variables in the management of CD patients, including fecal calprotectin, were recorded at baseline in all patients. All patients were Caucasian of Mediterranean ethnicity and were classified according to the Montreal classification [21]. All included patients received diaries to record symptoms 1 week prior to inclusion and sample collection.

Blood samples were obtained for routine hematological and biochemical studies at inclusion and inoculated in aerobic and anaerobic blood culture bottles, 10 ml each. Simultaneously, two separate blood samples were inoculated under aseptic conditions in rubber-sealed sterile Vacutainer SST II and K3E tubes, respectively (BD Diagnostics, Erembodegem, Belgium), which were never exposed to free air. Peripheral blood mononuclear cells (PBMCs) were isolated using Biocoll Separating Solution (Biochrom GmbH, Berlin, Germany) according to manufacturer's instructions.

Identification of bactDNA fragments and IL26 genotyping

Genomic DNA was isolated from 5×10^6 cells with the QIAmp DNA Blood Minikit (Qiagen, Hilden, Germany). BactDNA was identified by running a broad-range PCR with universal eubacterial primers of a conserved region of *16SrRNA* gene followed by partial nucleotide sequencing, as previously described [22].

We studied rs2870946, rs1558744, and rs7134599 SNPs in the *IL26* gene, located on chromosome 12q15. After extraction of genomic DNA from the peripheral blood with the QIAamp DNA Blood Mini Kit (Qiagen), partial amplification of the regions containing the different polymorphisms was performed using the following specific primers: rs2870946 forward 5′-GCTTAATTGCTCCAGCCATGC-3′, reverse 5′-GAAATGGGAAGGCACAGGCTA-3′; rs1558744 forward 5′-CTCCCACCCACCCCAATTTA-3′, reverse 5′-ACGG GTTGACCTGTTCAGAG-3′; and rs7134599 forward 5′-AGAAGTGAGCTTGCTTGTCTGT-3′, reverse 5′-GCAG GATCAAAATGTCAAGCAGT-3′. The PCR products, of 174, 182, and 153 bp length, respectively, were purified using

ExoSAP-IT PCR Product Cleanup (Affymetrix). The incidence of polymorphisms was detected by nucleotide sequencing of PCR products using the same primers as for the amplification. Subsequently, the sequencing process was performed in the sequencing service of Secugen S.L. The results were analyzed with FinchTV software version 1.5 (Geospiza). Patients bearing either one or more of the studied SNPs were grouped as variant (var) IL26 patients and compared with wild-type (wt) IL26 patients.

Serum cytokines, complement proteins and free anti-TNF-α levels. Presence of anti-drug antibodies

IL26 levels in serum samples were determined by handling an enzyme-linked immunosorbent assay (ELISA) for human IL26 measurement (Cloud-Clone Corp. Houston, TX) according to the manufacturer's instructions. Sensitivity assays were run to evaluate possible differences between wt and var*IL26*-genotyped patients and between IL26 either alone or in the presence of bactDNA (Supplementary Fig. 1). Serum TNF-α, IFN-γ, and IL-12p40 levels were determined by cytometric bead arrays (CBA) and measured with a FACSCanto II flow cytometer (Becton Dickinson, San Jose, CA).

ELISA kits were also carried out to measure free infliximab and adalimumab levels and to detect anti-drug antibodies (Matriks Biotek, Ankara, Turkey) according to the manufacturer's instructions. Proteins of the complement system C3b, the membrane attack complex (MAC), and C5a were evaluated by MicroVue EIA kits (Quidel Corporation, San Diego, CA) in serum samples of CD patients according to manufacturer's instructions, as previously described [23]. All samples were tested in triplicate and read in a Sunrise Microplate Reader (Tecan, Männedorf, Switzerland). The detection limits for each cytokine assay varied between 2 and 5 pg/mL, between 10 and 30 ng/mL in the case of free anti-TNF-α kits, and between 5 and 8 pg/mL for the complement protein assays. Standard curves were generated for every plate, and the average zero standard optical densities were subtracted from the rest of the standards and samples to obtain a corrected concentration for all parameters. The presence of anti-drug antibodies was evaluated by a cut-off value estimated by multiplying the optical density (OD) of the zero standard by 3, as indicated by the manufacturer's. Samples were considered positive when the ratio sample OD/zero standard OD was higher than 3.

Peripheral blood mononuclear cell killing assays

To evaluate blood PBMC bactericidal activity, cells from a subset of wt*IL26* and var*IL26* genotyped patients were isolated and seeded left untreated or treated with anti-IL26 or an anti-IgG2 isotype control (Sigma-Aldrich, Madrid, Spain) for a 48h period. Following this incubation, 2.5×10^6 cells were washed and exposed to the same amount of *E. coli* (10^4 CFU; serotype 0111:B4). The killing assay was performed at 37 °C in a shaker at 15 rpm for 20 min to measure the basal capacity of PBMCs to ingest bacteria before killing occurred (T0), or 2 h to measure the real PBMC killing ability (T2). Extracellular *E. coli* were thoroughly washed and subjected to a 30% sucrose centrifugation to better eliminate bacteria. Cells were resuspended in PBS 5% serum and then diluted in sterile water, to be seeded in agar plates at the estimated dilution ratio. Plates were left 24 h at 37 °C and colony-forming units counted afterwards.

Cell cultures

PBMCs from patients without bactDNA not receiving biologics or immunosuppressors were washed with phosphate-buffered saline (PBS) at 4 °C. Viability of isolated cells was evaluated by trypan blue staining (Sigma, Madrid, Spain). Cells were resuspended in phenol red-free RPMI 1640 medium (Gibco BRL, Life Technologies, Paisley, UK) supplemented with 10% human serum AB (BioWhittaker, Walkersville, Maryland, USA). PBMCs (1×10^6 cells/well) were incubated with the following: (a) infliximab (100 μg/mL/10^6 cells) (infliximab was kindly provided by Merck Sharp and Dohme of Spain MSD, Madrid, Spain) plus *E. coli* DNA (200 ng/mL/10^6 cells) for 48h; (b) recombinant human IL26 (R&D Systems) (100 ng/1×10^6 cells) plus *E. coli* DNA (200 ng/mL/10^6 cells) for 24 h.

Statistical analysis

Continuous variables were reported as mean ± standard deviation or 95% confidence interval, and categorical variables were expressed as frequencies and percentages. Differences between patient groups were analyzed using the *U*-Mann Whitney test for quantitative data and Chi-square test for qualitative data. The Kolmogorov–Smirnov test was used to test normality of continuous variables. Statistical differences were analyzed using the $\chi 2$ test for categorical data, and analysis of variance (ANOVA) test for quantitative data followed by the post hoc Bonferroni correction for multiple comparisons. Quantitative data showing non-normal distribution were analyzed using the Mann–Whitney *U* test or Kruskal–Wallis test followed by pairwise comparisons using the Mann–Whitney *U* test with the post hoc Bonferroni correction for multiple comparisons. Bivariate correlations were analyzed using the Spearman test. Statistical significance was considered at *p*-values less than 0.05. Statistical analysis was performed using SPSS v15 and R software.

Results

Characteristics of patients

Three hundred and thirteen CD patients in remission were included in the study. Clinical and analytical characteristics of patients are shown in Table 1. Briefly, mean age was 42 ± 15 years and 54% were male. Mean CDAI was 83.6 ± 32.8, and mean fecal calprotectin was 52.4 ± 34.8 μg/g. These parameters, along with ESR and CRP levels shown in Table 1, confirm clinical remission in our series of patients. Forty percent of patients were active smokers and 25% had a history of previous surgery. Ileal disease was present in 45% of patients, whereas colonic or ileo-colonic disease was present in 50% of patients. Twenty-one percent of patients presented with perianal disease. Twenty-eight percent of patients were on anti-TNF, either alone or combined, and 55% were receiving immunosuppressors.

BactDNA translocation and IL26 SNP distribution in CD patients

One hundred and six patients had bactDNA fragments in the blood (33.8%). Supplementary Table 1 presents sequencing analysis showing the identified bacterial species and the amount of amplified bactDNA for each species. BactDNA belonged to the *Enterobacteriaceae* family in 78% of patients. The amount of amplified bactDNA was not significantly different between species, family, or gram distribution.

IL26 SNP distribution of genotypes and allelic frequencies among included patients can be followed in Table 2. All variants were found to be in the Hardy–Weinberg equilibrium in the controls. Variant *IL26* (var*IL26*)-genotyped patients (n = 223) were defined as carrying any of the three variants studied either in homozygosis or heterozygosis compared with patients with a wild-type (wt) *IL26* genotype (n = 90). The rate of bactDNA translocation in var*IL26*-genotyped patients was similar to that present in wt*IL26*-genotyped patients (33.6 vs 34.4%, p = ns). No significant clinical or analytical differences were found among included CD patients distributed either by bactDNA translocation or *IL26* genotype (data not shown).

IL26 serum levels respond to systemic bactDNA translocation

The presence of circulating bactDNA was associated with significantly increased levels of IL26 regardless of *IL26* genotype status compared with bactDNA-negative patients (101.6 ± 25.4 vs 18.9 ± 11.2, p = 0.001). This elevation was blunted in patients with var*IL26* genotype (Fig. 1a).

The amount of bactDNA was significantly increased in var*IL26* vs wt*IL26* patients (26.9 ± 6.0 vs 30.8 ± 7.2, p = 0.01). The overall correlation between amplified bactDNA concentration in patients with bactDNA and IL26 serum levels was statistically significant although weak (r = 0.37; p = 0.01). This correlation significantly improved when only wt*IL26* patients were considered (r = 0.77; p = 0.001). The correlation between both variables was lost when patients with a var*IL26* genotype (r = 0.08; p = 0.476) were considered (Fig. 1b). These results were confirmed in vitro in PBMCs from wt and var*IL26* patients exposed to different concentrations of *E. coli* bactDNA (Fig. 1c).

Functional capacity of PBMCs to kill bacteria is decreased in varIL26-genotyped patients

These results suggest that PBMC interaction with bacteria is compromised in var*IL26*-genotyped patients. To confirm this, killing capacity of PBMCs from a subset of bactDNA-negative patients was evaluated. Figure 2 shows that wt*IL26*-genotyped patients' PBMCs are able to significantly reduce the total CFU counts 2 h after exposure to *E. coli.* However, the presence of a var*IL26* genotype significantly worsens PBMC killing capacity, as total CFU count significantly increases at 2 h in comparison to wt*IL26*-genotyped PBMCs. In addition, preincubation of PBMCs from wt*IL26* and var*IL26*-genotyped patients with anti-IL26 further reduces PBMC killing capacity.

The soluble inflammatory response to bactDNA is sensitive to IL26 genotype

We then evaluated the soluble inflammatory response to bactDNA in patients distributed by *IL26* genotype. Serum levels of proinflammatory mediators in patients without bactDNA were as follows: TNF-α [44.4 ± 29.8 pg/mL], IFN-γ [102.2 ± 68.5 pg/mL], and IL-12 [194.3 ± 70.4 pg/mL]. These levels significantly increased both in wt*IL26* and var*IL26* patients with bacterial DNA (Fig. 3a). However, the variant genotype was associated with significantly further increased levels of all three cytokines compared to wt*IL26*-genotyped patients with bactDNA. To support this result, serum levels of complement proteins were measured and showed a significant increase in the overall series of patients with vs without bactDNA (Supplementary Table 2). The presence of a var*IL26* further increased complement protein levels in patients with bactDNA compared to wt*IL26*-genotyped patients.

We then cultured PBMCs from bactDNA-negative patients distributed by *IL26* genotype with human recombinant IL26 and *E. coli* DNA. Cytokine levels were

Table 1 Clinical and analytical characteristics of patients

	Patients (n = 313)
Age (years)	42 ± 15
Weight (kg)	70.24 ± 14.86
Gender (male/female), *n* (%)	167 (53.4%)/146 (46.6%)
Smoking habit (yes/no/ex), *n* (%)	126 (40.2%)/119 (38.0%)/68 (21.8%)
Disease duration (months)	115.15 ± 114.55
Resection, *n* (%)	71 (22.6%)
CDAI	83.6 ± 32.8
Montreal A (age of onset), *n* (%)	
A1 (≤ 16)	18 (5.8%)
A2 (17–40)	219 (70.0%)
A3 (> 40)	76 (24.2%)
Montreal L (location), *n* (%)	
L1	143 (45.6%)
L2	70 (22.3%)
L3	86 (27.4%)
L4	14 (4.5%)
Montreal B (behavior), *n* (%)	
B1 (non-stricturing, nonpenetrating)	151 (48.2%)
B1p (non-stricturing, nonpenetrating, penetrating perianal disease)	40 (12.8%)
B2 (stricturing)	49 (15.6%)
B2p (stricturing, perianal disease associated)	14 (4.5%)
B3 (penetrating)	45 (14.4%)
B3p (penetrating, penetrating perianal disease)	14 (4.5%)
Therapy, *n* (%)	
Mesalazine	70 (22.4%)
Azathioprine	104 (33.2%)
Metotrexate	9 (2.9%)
Mesalazine and azathioprine	17 (5.4%)
Mesalazine and steroids	4 (1.3%)
Azathioprine + steroids	4 (1.3%)
Metotrexate + steroids	1 (0.3%)
Infliximab	27 (8.6%)
Adalimumab	29 (9.3%)
Infliximab + azathioprine	16 (5.1%)
Adalimumab + azathioprine	11 (3.5%)
Infliximab + steroids	2 (0.6%)
Adalimumab + steroids	4 (1.4%)
Infliximab + metotrexate	2 (0.6%)
Infliximab + azathioprine + steroids	1 (0.3%)
No therapy	12 (3.8%)
CRP (mg/dL)	0.58 ± 1.10
Fecal calprotectin (μg/g)	52.40 ± 34.80
ASCAs, *n* (%)	118 (39.6%)
Hemoglobin (g/dL)	13.95 ± 2.35
ESR (mm)	19.3 ± 15.4
Albumin (g/dL)	4.12 ± 4.50
Total WBCs (mm^3)	6892.5 ± 2636.8
Temperature (°C)	36.07 ± 0.22
Pulse rate (bpm)	70.68 ± 6.11

Table 2 Allelic frequencies of *IL26* SNPs in included CD patients

Genotype *n* (%) patients/controls				
	Homozygous wild-type	Heterozygous	Homozygous variant	Variant allele frequency (%)
IL26 rs1558744 (A > G)	55 (17.5%)/88 (17.5%)	178 (56.8%)/229 (45.5%)	80 (25.5%)/186 (37.0%)	53.9/59.7
IL26 rs2870946 (T > C)	243 (77.6%)/431 (85.7%)	67 (21.4%)/68 (13.5%)	3 (0.9%)/4 (0.8%)	11.6/7.6
IL26 rs7134599 (G > A)	88 (28.1%)/208 (41.4%)	170 (54.3%)/76 (15.1%)	55 (17.5%)/219 (43.5%)	44.7/36.9

significantly higher in the supernatants of PBMCs cultured with bacterial DNA from var*IL26* patients than in wt*IL26* patients (white vs black bars), supporting the in vivo results. The addition of the recombinant IL26 protein was associated with a reduction in pro-inflammatory cytokine levels in the supernatants of cultured var*IL26* cells to levels present in wt*IL26* cells in response to *E. coli* DNA (Fig. 3b). Supplementary Table 3 shows cytokine levels in the supernatants of cultured PBMCs unstimulated and with human recombinant IL26 alone.

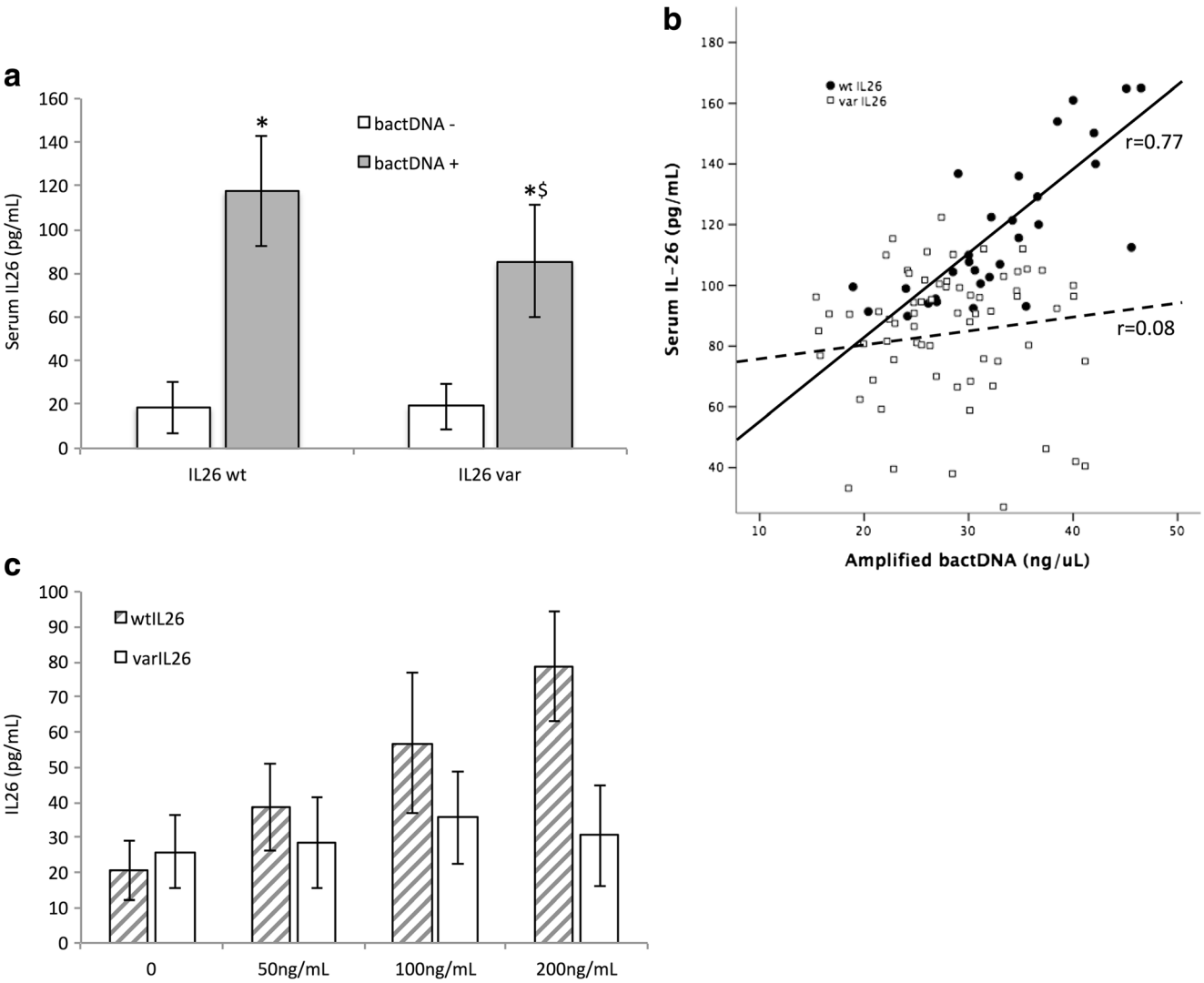

Fig. 1 Serum IL26 levels in CD patients. **a** Serum IL26 levels in CD patients distributed by *IL26* genotype and the presence of bacterial DNA in blood. **b** Correlation between IL26 serum levels and amplified bactDNA in patients with bactDNA in blood distributed by *IL26* genotype. *$p < 0.01$ compared with patients without bactDNA; $^{\$}p = 0.01$ compared with wt*IL26* with bacterial DNA. **c** IL26 levels in the supernatants of PBMCs from wild-type and variant *IL26*-genotyped patients cultured with increasing amounts of *E. coli* DNA. Figure shows mean ± SD from 10 patients from each group. *$p < 0.01$ compared with wt*IL26*. *wt* wild-type, *var* variant

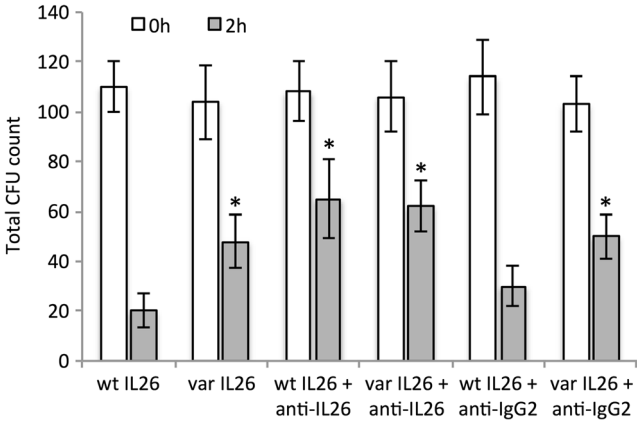

Fig. 2 *E. coli* killing assays on blood PBMCs. Total CFUs before ($T = 0$) and 2 h after *E. coli* exposure ($T = 2$) are represented. Results were obtained in experiments using 2.5×10^6 cells from 10 wt*IL26* and 10 var*IL26* patients. *$p < 0.01$ compared with wt*IL26* patients and wt*IL26* patients incubated with an IL26 isotype control

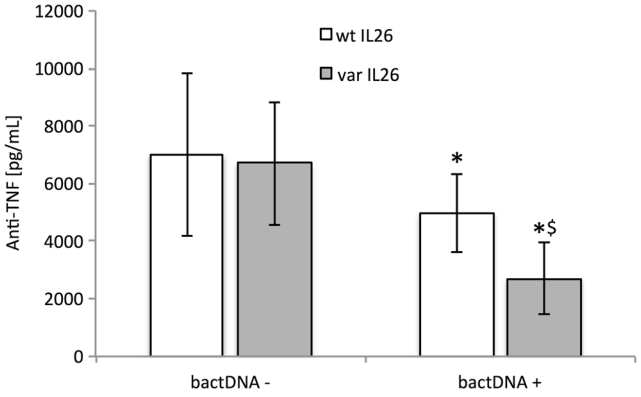

Fig. 4 Free anti-TNF-α serum levels in CD patients on biologics distributed by the *IL26* genotype and the presence of bactDNA. *$p < 0.01$ compared with levels in patients without bacDNA. $^{\$}p < 0.01$ compared with levels in wt*IL26*-genotyped patients

Anti-TNF treatment efficacy is affected by IL26 genotype in patients with bacterial DNA

As var*IL26* was associated with elevated TNF-α concentrations in patients with circulating bactDNA, we studied var*IL26* effects on the treatment with anti-TNF-α drugs. Six out of 92 patients on biologics (4 wt*IL26* and 2 var*IL26*) had anti-drug antibodies in the blood and were excluded from this analysis. Figure 4 shows that serum free anti-TNF-α levels in var*IL26*-genotyped patients were significantly lower than levels in wt*IL26*-genotyped patients in the presence of bactDNA.

To confirm the further reduction of serum free anti-TNF-α levels associated with a var*IL26* genotype and the presence of bactDNA, we cultured PBMCs from bactDNA-negative patients distributed by *IL26* genotype with *E. coli* DNA and infliximab (Fig. 5). While TNF-α levels significantly increased, the concentration of infliximab significantly decreased in the supernatant of cultured cells from patients with a var*IL26* genotype stimulated with *E. coli* DNA compared with wtIL26-genotyped patients. IL26 blockade in wt*IL26*-genotyped patients further increased TNF-α levels in the supernatants and signficantly reduced free anti-TNF-α levels. On the contrary, IL26 supplementation to PBMCs from var*IL26* patients resulted in the partial restoration of the TNF-α response and available anti-TNF-α levels shown by wt*IL26*-genotyped patients.

A varIL26 genotype is associated with the initiation of anti-TNF-α therapy in CD patients in remission

Patients were followed up for 6 months. None of the patients were lost during the follow-up period. Forty patients relapsed in this time frame, with no differences according to *IL26* genotype (11 of 90 wtIL26 [12.2%] patients vs 35 of 223 [15.7%] var*IL26* patients, $p = 0.104$). Figure 6 shows the

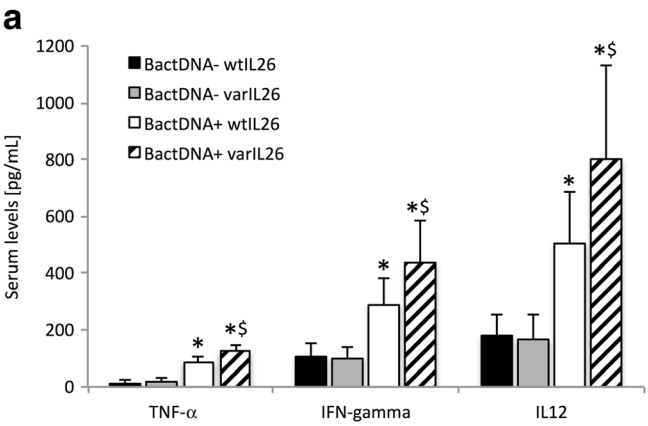

Fig. 3 **a** Serum levels of TNF-α, IFN-γ, and IL-12p40 in CD patients with bactDNA distributed by *IL26* genotype. *$p < 0.01$ compared with serum levels in patients without bactDNA; $^{\$}p < 0.01$ compared with serum levels in bactDNA + wt*IL26* patients. **b** Levels of TNF-α, IFN-γ, and IL-12p40 in the supernatants of PBMCs from bactDNA-negative patients either with recombinant human IL26 or not and cultured with *E. coli* DNA (see methods). Results were obtained in experiments using 1×10^6 cells from 10 wt*IL26* and 10 var*IL26* patients. *$p < 0.01$ compared with the rest of conditions

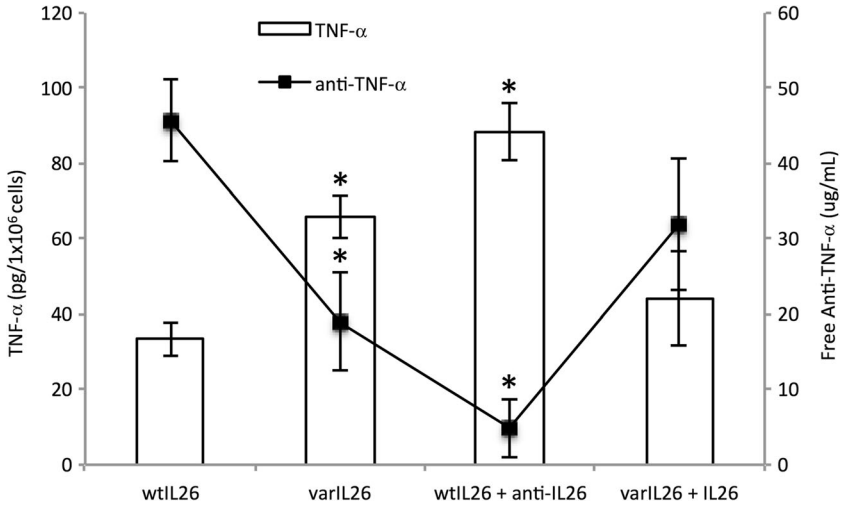

Fig. 5 In vitro analysis of TNF-α and free anti-TNF-α levels in the supernatants of PBMCs from wt*IL26* and var*IL26* patients cultured with *E. coli* DNA (200 ng/mL/10^6 cells) plus infliximab (100 μg/mL/10^6 cells) in all cases. Results were obtained in experiments using 1×10^6 cells from 10 wt*IL26* and 10 var*IL26* patients. *$p < 0.01$ compared with wt*IL26*

percentage of patients who required either initiation, intensification, or switch of biologics. The initiation of biologics, the need for intensification, and switch of biologic drug were more frequent in var*IL26*-genotyped patients. However, the initiation of biologics was the only statistically significant variable between groups.

Discussion

The present study shows that IL26 is sensitive to bactDNA translocation into blood of CD patients. In addition, a var*IL26* genotype is associated with reduced IL26 serum levels in patients with this circulating antigen and the reduction of PBMC functional capacity to kill bacteria. This might compromise bactDNA clearance, inducing a sustained pro-inflammatory environment, and an increased anti-TNF-α consumption.

Bacterial DNA translocation has been identified as a risk factor of relapse in CD patients in remission. In addition to this, an increased risk of other significant clinical events such as the inititation of steroids or the switch to stepped up treatments has also been associated with the detection of circulating bacterial genomic fragments in the blood of CD patients [9]. Likely, the presence of circulating bactDNA induces the secretion of pro-inflammatory cytokines leading to an increased [1] and dose-dependent production of antimicrobial peptides [24]. Its interaction with the immune system is especially relevant in patients with *NOD2* gene variants, who seem to be predisposed to CD development [25, 26] and may show an impaired response against the translocation of commensal bacterial antigens [8]. These data support that the translocation of bactDNA may be a risk factor for a more severe disease course.

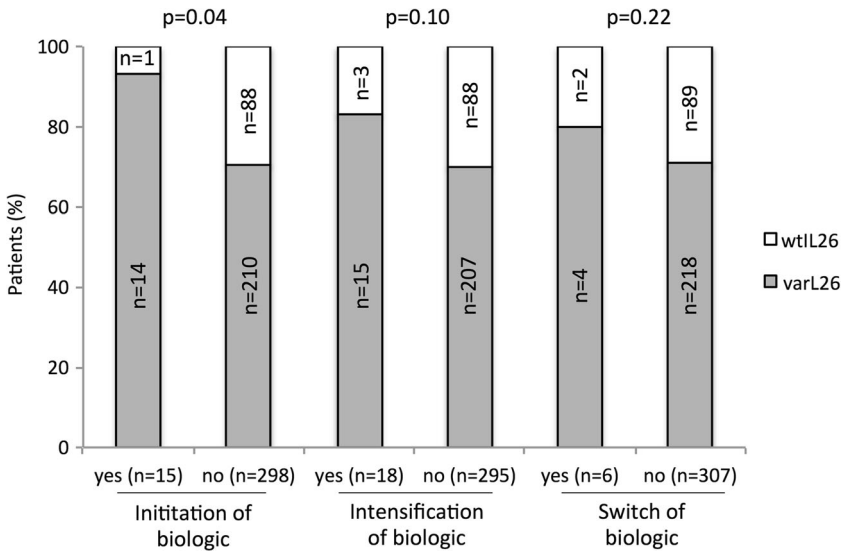

Fig. 6 Clinical evolution of patients. Initiation, intensification, and switch of biologics among included CD patients during follow-up are represented

IL26, a member of the IL-20 cytokine family and part of the Th17 cell cytokines, has been shown to assemble with bactDNA fragments and promote TLR-9 activation in antigen presenting cells to deliver a pro-inflammatory immune response [14]. This facilitates the clearance of circulating bactDNA and may prevent a sustained inflammatory environment. Several *IL26* SNPs have been described, and we hypothesized that a var*IL26* genotype may affect IL26 interaction with bactDNA and impair the required immune response to resolve this bacterial antigen challenge.

To our knowledge, this is the first study evaluating the effect of *IL26* SNPs in CD patients. We have observed that IL26 levels are significantly increased in patients with bactDNA. However, the presence of a var*IL26* genotype in patients with bactDNA is associated with significantly reduced IL26 levels compared with wt*IL26* patients (Fig. 1). Although a limitation due to untested effect of IL26-DNA complexes on measured IL26 serum levels may be considered, these results suggest first that IL26 production depends in part on the presence of circulating bactDNA in CD patients. In fact, a positive correlation between IL26 and the amount of amplified bactDNA is present in wt*IL26*-genotyped patients, indicating that this cytokine participates in defense programs against bacterial challenges mediated by Th17 responses [27, 28]. Secondly, that the clearance of bactDNA in these patients may be affected by the presence of a var*IL26* genotype, which may partially explain the observed genetic susceptibility to CD in patients with SNPs in the *IL26* region [16]. In addition, the concentrations of the pro-inflammatory cytokines TNF-α, IFN-γ, and IL-12 are significantly increased in bactDNA-positive CD patients with a var*IL26* genotype (Figure 3a). Potentially, the increased exposure of bactDNA could stimulate the innate immune machinery, in which activated macrophages are recruited, the complement cascade is initiated, and endothelial cells are activated to induce TNF-α and other inflammatory mediators that further contribute to sustain an upheld pro-inflammatory environment [29]. In agreement with this, complement proteins are significantly increased in response to bactDNA in patients with a var*IL26* genotype compared to patients with a wt*IL26* genotype.

The relevance of an increased systemic immune response in CD patients may be reflected in their need for intensified anti-TNF-α therapy. In the present investigation, the significant reduction in serum TNF-α levels in patients on biologics was only achieved in wt*IL26*-genotyped patients. In consequence, we found that free anti-TNF-α trough levels are significantly reduced in var*IL26* patients (Fig. 4). To model the in vivo situation in vitro, PBMCs from patients without bactDNA were stimulated with *E. coli* in the presence of infliximab. Cultures of PBMCs from var*IL26* patients showed significantly lower amounts of free anti-TNF-α of PBMCs from wt*IL26* patients (Fig. 5).

From a clinical point of view, the classification of CD patients by their *IL26* genotype could be of interest, not only in terms of their predisposition to an exacerbated inflammatory response, as they may be exposed to longer antigen circulation in the case of bacterial translocation, but also for the identification of patients who might respond worse to established drug schedules. In fact, the percentage of patients who required initiation of anti-TNF-α was significantly higher in var*IL26* vs wt*IL26*-genotyped patients (Fig. 6). Also, an increased percentage of var*IL26* patients included in our series, compared to wt*IL26* patients, required intensification or switch of biologic drug, although probably larger series of patients would be necessary to find a statistically significant association. Although results in this regard are merely exploratory and present several limitations that would require a specifically designed study, these patients might benefit from early therapies in the so-called top-down approach [30, 31], especially those subgroups with translocation of bacterial DNA, *NOD2*, or other CD-related gene variants.

In summary, IL26 participates in the modulation of the pro-inflammatory soluble response in CD patients in remission with bactDNA translocation. However, the presence of a var*IL26* genotype is associated with a reduced PBMC functional capacity to kill bacteria, the stimulation of an exacerbated inflammatory response, and the increment of anti-TNF-α consumption in these patients. These results may help in explaining the identification of bactDNA in blood as an independent risk factor of flare at short term in CD patients. Further studies on IL26 signaling restoration may provide new therapeutic approaches to control bacterial translocation and inflammation in this subgroup of CD patients.

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Conflict of interest The authors declare that they have no conflicts of interest.

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