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Regulatory T Cells in Patients with Whipple's Disease

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Classical Whipple's disease (CWD) is caused by chronic infection with *Tropheryma whipplei* that seems to be associated with an underlying immune defect. The pathognomonic hallmark of CWD is a massive infiltration of the duodenal mucosa with *T. whipplei*-infected macrophages that disperse systemically to many other organ systems. An alleviated inflammatory reaction and the absence of *T. whipplei*-specific Th1 reactivity support persistence and systemic spread of the pathogen. In this article, we hypothesized that regulatory T cells (T_{reg}) are involved in immunomodulation in CWD, and we asked for the distribution, activation, and regulatory capacity of T_{reg} in CWD patients. Whereas in the lamina propria of CWD patients before treatment numbers of T_{reg} were increased, percentages in the peripheral blood were similar in CWD patients and healthy controls. However, peripheral T_{reg} of CWD patients were more activated than those of controls. Elevated secretion of IL-10 and TGF-β in the duodenal mucosa of CWD patients indicated locally enhanced T_{reg} activity. Enhanced CD95 expression on peripheral memory $CD4^+$ T cells combined with reduced expression of IFN-γ and IL-17A upon polyclonal stimulation by $CD4^+$ cells from untreated CWD patients further hinted to T_{reg} activity-related exhaustion of effector $CD4^+$ T cells. In conclusion, increased numbers of T_{reg} can be detected within the duodenal mucosa in untreated CWD, where huge numbers of *T. whipplei*-infected macrophages are present. Thus, T_{reg} might contribute to the chronic infection and systemic spread of *T. whipplei* in CWD but in contrast prevent mucosal barrier defect by reducing local inflammation. ***The Journal of Immunology*****, 2011, 187: 4061–4067.**

Classical Whipple's disease (CWD), first described by G.H. Whipple in 1907 (1), is a chronic multisystemic infection with *Tropheryma whipplei* (2, 3) with major symptoms in the joints and in the intestinal tract (4, 5). Massive infiltration of the duodenal mucosa by macrophages that are filled with huge amounts of *T. whipplei* is the pathognomonic hallmark of CWD. The disease is fatal if not treated with appropriate antimicrobials (6). The low incidence of CWD despite the ubiquitous presence of *T. whipplei* in the environment and in control persons (7, 8) as well as effective humoral and cellular immunological responses against the agent in healthy adults (9, 10) indicate predisposing immunological defects in patients with CWD. Several immunological aberrations have been found so far in patients with CWD, most of them persisting also after successful eradication of *T. whipplei* (10–14). Although an impairment of unspecific proliferation of T cells (15) and a weak IL-12 secretion (11, 12) have been described only for the peripheral blood, a reduced $CD4^+$:$CD8^+$ T cell ratio (15), diminished Th1 reactivity (13), absent *T. whipplei*-specific Th1 response (10), and alternative macrophage activation (14, 16) are likewise apparent in lymphocytes of the duodenal mucosa. In a previous study, it was shown that in the duodenum and the peripheral blood proinflammatory cytokines such as IL-1β and TNF-α are not enhanced in CWD patients compared with healthy controls, whereas an elevated expression of IL-10 of CWD patients may facilitate the chronic infection with *T. whipplei* (14). Hence, the immunological reaction of the intestinal mucosa seems to influence the pathogenesis of CWD.

The immunological balance of the gut is particularly important to prevent inflammation, which would disturb the mucosal barrier function. Consequently, regulatory T cells (T_{reg}), characterized by a high expression of CD25 and the transcription factor FOXP3 (17, 18), are important players especially for the intestinal homoeostasis. They have been shown to be crucial in chronic infections of the gut with, for example, *Helicobacter pylori* (19–21) and are thought to reduce tissue damage induced by HIV or *Yersinia enterocolitica* (22–24). IL-10 and TGF-β are the major cytokines involved in mucosal T cell regulation and impaired secretion favors pathogen-induced tissue damage (20, 24).

The role of T_{reg} in CWD has not been studied so far. Hence, we assessed T_{reg} in the duodenal mucosa of CWD patients compared with healthy controls. Furthermore, T_{reg} in the peripheral blood were characterized, based on the expression of different markers of activity, and the regulatory capacity of T_{reg} was analyzed functionally ex vivo.

Materials and Methods

Patients and control subjects

Samples from 93 patients with CWD (Table 1; 15 with untreated disease, 41 treated, 37 before and after treatment) and 122 control subjects without detectable *T. whipplei* were studied (Table I). Gastrointestinal symptoms were present in 88 CWD patients; 3 patients had isolated neurologic symptoms and 2 only articular manifestation. The diagnosis of CWD was confirmed by at least two tests (5). The majority of patients were treated

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The online version of this article contains supplemental material.

Abbreviations used in this article: CBA, cytometric bead array; CWD, classical Whipple's disease; PD-1, programmed death-1; SEB, staphylococcal enterotoxin B; T_{reg}, regulatory T cell; T_{resp}, responder T cell.

for 2 wk with ceftriaxone, followed by either 12 or 3 mo of trimethoprime–sulfamethoxazole; two patients received only trimethoprime–sulfamethoxazole; four patients were alternatively treated with doxycycline, two of them in combination with hydroxychloroquine and the other with IFN-γ. Total remission was achieved in 90 patients, whereas 3 patients had persisting problems because of irreversible tissue damage. Blood from 47 healthy subjects and 43 CWD patients was collected in sodium-heparinized and serum tubes (Vacutainer; BD Biosciences) and processed within 24 h. Serum samples from 28 healthy subjects and 31 CWD patients were stored at −20°C. Duodenal biopsies were analyzed from 67 CWD patients and 62 biopsies without pathological findings from patients examined either for control of former ulcer disease or for differential diagnosis of unspecific gastrointestinal or unexplained rheumatic signs. Duodenal biopsies where cultured for the assessment of cytokine production or were fixed in 4% paraformaldehyde (Sigma-Aldrich) for immunohistochemical analysis.

Permission for the study was obtained from the Clinical Ethics Committee of the Charité, and all subjects gave their written consent to participate.

Immunohistochemistry

Immunohistochemical staining on paraffin sections of duodenum specimen was performed as described previously (22). Rat anti-human FOXP3 (dilution 1/200, clone PCH101; eBioscience) was used as primary Ab, and the staining was visualized by rabbit anti-rat biotin (dilution 1/200; Jackson ImmunoResearch Laboratories), streptavidin–alkaline phosphatase (dilution 1/400; Sigma-Aldrich), and Fast Red (DakoCytomation). Nuclei were counterstained with Meyer's hematoxylin (DakoCytomation). Positive cells were quantified in biospies per high-power field (0.237 mm^2), and 10–20 high-power field were averaged in each case. All immunohistochemical evaluations were performed in a blinded manner.

Short-term culture of intestinal biopsy samples

Culture supernatants of duodenal biopsy samples were prepared as described previously (14). Biopsy samples were placed immediately after surgery into PBS (PAA Laboratories), washed, weighted, and incubated on a metal mesh covered with RPMI 1640 medium (Invitrogen) containing 10% FCS (Sigma-Aldrich), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin (Biochrom) on a shaking platform for 48 h at 37°C in a humidified 5% CO_2/80% O_2 atmosphere. Supernatants were stored at −80°C until assay.

Analysis of cytokine secretion in serum and biopsy cultures

Concentrations of IFN-γ and IL-10 (BD Biosciences) as well as of TGF-β (Bender MedSystems) in serum samples and in supernatants of biopsy cultures were quantified by cytometric bead arrays (CBA), according to the manufacturer's protocols.

Bacterial strains and preparations

T. whipplei Twist Marseille (CNCM I-2202) (3) was cultured in axenic medium as described previously (25). Bacteria were heat killed, sonicated, and used for *T. whipplei*-specific stimulation.

Isolation of peripheral cell populations

PBMC were separated by Ficoll-Paque (GE Healthcare) in Leucosep tubes (Greiner Bio-One) and washed twice in PBS containing 0.5% BSA (Sigma-Aldrich). $CD4^+$ T cells and $CD4^{-/low}$ cell populations containing APC, $CD25^+CD4^+$ T_{reg}, and $CD25^-CD4^+$ responder T cell (T_{resp}) populations were isolated from PBMC using the $CD4^+CD25^+$ Regulatory T Cell Isolation Kit (Miltenyi Biotec), according to the manufacturer's protocol. In the first step, $CD4^+$ T cells were sorted by negative selection with a purity of 93 ± 2% $CD4^+$ (Supplemental Fig. 1*A*, 1*C*). In the second step, T_{reg} were obtained by positive selection with a typical yield of 0.6–8.8 × 10^5 T_{reg} per 10^8 PBMC and a purity of 55 ± 4% $CD4^+CD25^{high}$ (Supplemental Fig. 1*B*, 1*C*).

CFSE labeling

Cells were washed in PBS, suspended at 1 × 10^7/ml in PBS containing 0.5 μM/ml CFSE (Molecular Probes), incubated for 3 min at room temperature, and washed twice with culture medium. The viability of the labeled cells as determined by trypan blue (Sigma-Aldrich) exclusion was >95%.

Assessment of T_{reg} activity and specificity in vitro

T cell suppression assays were performed in RPMI 1640 with glutamax (Invitrogen) supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 25 mM HEPES (PAA Laboratories) in a humidified 5% CO_2 air atmosphere at 37°C.

To measure the general suppressive capacity, sorted T_{reg} (Supplemental Fig. 1*B*, 1*E*) from treated CWD patients and healthy controls were added at different ratios (1/16, 1/8, 1/4, 1/2; results are only presented for 1/2) to 5 × 10^4 CFSE-labeled T_{resp} in 96-well U-bottom plates (Nunc) coated with 0.6 μg/well anti-CD3 Ab (SP34/r; BD Biosciences) and incubated in the presence of 1 μg/ml soluble anti-CD28 Ab (CD28.2 NA/LE; BD Biosciences). T_{reg} applied in the assay revealed a stable regulatory phenotype as assessed by the maintenance of a high CD25 and FOXP3 expression after 4 d of cultivation with supplementation of recombinant human IL-2 (20 U/ml; R&D Systems) (Supplemental Fig. 1*E*).

To investigate the specificity of the suppressive activity, the response to heat-inactivated *T. whipplei* was determined after depletion of T_{reg}. CFSE-labeled T_{resp} (5 × 10^4; Supplemental Fig. 1*B*, 1*C*) or total $CD4^+$ T cells (5 × 10^4, Supplemental Fig. 1*A*, 1*C*), were cocultured with 1 × 10^4 $CD4^{-/low}$ cells containing APC (30 ± 2% $HLA\text{-}DR^+$; Supplemental Fig. 1*A*, 1*D*) in the presence of *T. whipplei* (10^7/ml) and soluble anti-CD28 Ab (1 μg/ml) for 4 d. Brefeldin A (10 μg/ml; Sigma-Aldrich) was added for the last 4 h before the assessment of CFSE dilution for proliferation and intracellular IFN-γ by flow cytometry. The background (values of control without stimulus) was deducted from values for the specific stimulations, and values below background level were defined as 0.

Evaluation of T cell activity from whole blood specimen

To determine the general Th1 and Th17 reactivity, fresh heparinized blood was incubated as previously described (10) without (negative control) or with staphylococcal enterotoxin B (SEB; 2 μg/ml; Sigma-Aldrich) for 6 h at 37°C in a humidified 5% CO_2 air atmosphere. Brefeldin A (10 μg/ml) was added for the last 3 h (to assess the cytokine production intracellularly), followed by lysis of erythrocytes and fixation. Cells were washed and stored in PBS containing 0.5% BSA and 0.02% NaN_3 (Merck) before intracellular staining and flow cytometric analysis. Percentages of activated Th1 ($IFN\text{-}\gamma^+CD40L^+CD4^+$) or Th17 ($IL\text{-}17A^+CD40L^+CD4^+$) in the negative controls were subtracted from the respective values obtained after stimulation with SEB (Supplemental Fig. 3*B*). Values after stimulation below background level were defined as 0.

Flow cytometric analysis

The following Abs were used: CD3 PerCP (SK7), CD4 PerCPCy5.5 (SK3), CD4 Pacific Blue (RPA-TA), CD25 FITC (M-A251) and CD25 allophycocyanin-H7 (M-A251), CD45RO PE-Cy7 (UCHL-1), CTLA-4 PE (B13), programmed death-1 (PD-1) FITC (CD279; MIH4), CD95 PE-Cy5 (DX2), CD40L (CD154) FITC (TRAP1), CD14 PerCP-Cy5.5 (M5E2), CD86 allophycocyanin (2331(FUN-1)), CD19 FITC (HIB19), and IFN-γ allophycocyanin (B27) from BD Biosciences; CD25 allophycocyanin (4E3) and CD39 allophycocyanin (MZ18-23C8) from Miltenyi Biotec; FOXP3 PE (PCH101) and IL-17A Alexa Fluor 647 (eBio64DEC17) from eBioscience; CD4 PE (MT310) from DakoCytomation and GITR allophycocyanin (110416) from R&D Systems; and HLA-DR PE (Immu357) from Beckman Coulter.

For staining of whole blood, Abs were added to 100 μl heparinized blood, incubated for 15 min at room temperature, followed by lysis of erythrocytes and fixation as described previously (10).

Cell suspensions were stained in 50 μl PBS containing Abs and 2% Beriglobin for 15 min at 4°C, followed by washing in PBS, and fixation in 4% paraformaldehyde for 10 min at 37°C. Intracellular staining of cytokines was performed after fixation in the presence of 0.5% saponin (Sigma-Aldrich).

For seven-color analysis, 1 ml fresh heparinized blood was incubated in 5 vol erythrocyte lysis Buffer (Qiagen) for 15 min on ice and subsequently washed once in erythrocyte lysis Buffer and twice in PBS/0.5% BSA/2 mM EDTA (PBE; Sigma-Aldrich) to remove erythrocytes. Abs were added in 100 μl PBE containing 2% Beriglobin (Behring), and cells were incubated for 15 min on ice, followed by washing in PBE. Intranuclear staining of FOXP3 was conducted using the FOXp3 Staining Set (eBioscience), according to the recommended procedure. Cells were resuspended in 1× BD CellFIX (BD Biosciences) and analyzed within 48 h.

Data acquired on a FACSCalibur with the CellQuest software or a FACSCanto II with the FACSDiva software (all BD Biosciences) were analyzed using the FlowJo software (Tree Star). At least 5 × 10^4 $CD4^+$ cells (four-color stain) or 10^5 lymphocytes as defined by granulation (side scatter) and particle size (forward light scatter) (seven-color stain) were investigated.

Statistical analysis

Quantitative parameters are presented as individual data points with median or indicated in the text as mean and SEM. Data were analyzed using the

GraphPad Prism5 software (GraphPad) by means of the Mann–Whitney *U* test or the Welch correction for different variances of the analyzed groups (IL-17A in whole-blood stimulation). The p values ≤ 0.05 were considered significant.

Results

High numbers of T_{reg} in the duodenal mucosa of CWD patients

To determine T_{reg} at the site of *T. whipplei* infection, FOXP3$^+$ cells were stained immunohistochemically in duodenal biopsies from CWD patients (Table I) with different stages of the disease and control subjects with no sign of *T. whipplei* infection (Fig. 1).

Patients with untreated CWD showed increased numbers of FOXP3$^+$ T_{reg} in the lamina propria as compared with control subjects. After treatment with antibiotics, the T_{reg} numbers in CWD patients significantly decreased even below the level of controls.

Regulatory phenotype of CD4$^+$ T cells in the peripheral blood of CWD patients

We analyzed the phenotype of CD4$^+$ T cells in the whole blood of CWD patients before and after antibiotic treatment and healthy subjects by flow cytometry to investigate T_{reg} in the peripheral blood of CWD patients.

As distinguished from duodenal tissue specimen, portions of T_{reg} in the periphery, characterized as CD25^{high} (Fig. 2*A*, Supplemental Fig. 2*A*) or CD25$^+$FOXP3$^+$ within the CD4$^+$ T cells (Fig. 2*B*, Supplemental Fig. 2*B*), did not differ in patients with both untreated and treated CWD and in healthy controls.

However, CD25^{high}CD4$^+$ T_{reg} in the peripheral blood of CWD patients showed significantly augmented surface expression levels of CTLA-4 (Fig. 2*C*, Supplemental Fig. 2*A*) and of CD39 (Fig. 2*D*, Supplemental Fig. 2*C*), which are both associated with enhanced regulatory activity (26–29). Although the expression levels of CTLA-4 on CD25^{high}CD4$^+$ T_{reg} declined upon treatment of CWD, the elevated expression levels of CD39 in CWD compared with healthy controls were maintained after the antibiotic therapy.

Anti-inflammatory cytokine milieu in CWD patients

As the cytokine milieu shapes the quality of T cell responses and might be indicative for the observed regulatory T cell phenotype, we assessed pro- and anti-inflammatory cytokines in the serum and in the supernatants of duodenal biopsies by CBA.

The amount of proinflammatory IFN-γ mainly produced by peripheral blood T cells was lower in CWD patients than in healthy controls (Fig. 3*A*). TGF-β essential for the induction of T_{reg} (30, 31) was highly increased in the serum of CWD patients independent of the antibiotic therapy (Fig. 3*B*). An increased production of IL-10 and TGF-β was also detected in cultures of duodenal biopsies from CWD patients as compared with supernatants of biopsies from control subjects (Fig. 3*C*, 3*D*). The levels of proinflammatory IL-17A in supernatants of duodenal biopsies were not significantly different in CWD patients and healthy subjects (CWD, 45.8 ± 24.9 pg/100 mg biopsy; controls, 12.45 ± 2.9 pg/100 mg biopsy).

T_{reg} activity in CWD is enhanced but not specific to T. whipplei

To test the functionality of T_{reg} in CWD patients, we compared the suppressive activity of CD25$^+$CD4$^+$ T_{reg} from the peripheral blood of CWD patients and of healthy control subjects toward the activation of T_{resp} in vitro. In this study, T_{reg} from treated CWD patients or healthy controls were added to autologous CFSE-

Table I. Investigated samples from patients with CWD and controls

	CWD	Control Subjects
Total	Samples from 93 CWD patients (18 F, 75 M; mean age 57 y; range 33–84) 37 patients before and after treatment 15 untreated patients 41 treated patients in remission	Samples from 122 control subjects (47 F, 75 M; mean age 42.1 y; range 22–84)
Blood		
Serum/plasma	Samples frp, 31 CWD patients (3 F, 28 M; mean age 57.3 y; range 42–74) 17 patients before and after treatment 10 untreated patients 4 treated patients in remission	Samples from 28 control subjects (17 F, 11 M; mean age 47.3 y; range 35–70)
Heparinized blood	Samples from 43 CWD patients (7 F, 36 M; mean age 56.8 *y*; range 41–77) 10 patients before and after treatment 8 untreated patients 25 treated patients in remission	Samples from 47 control subjects (26 F, 21 M; mean age 47.5 *y*; range 18–84)
Duodenal biopsies		
Total biopsies	Samples from 67 CWD patients (12 F, 55 M; mean age 57.3 y; range 33–84) 21 patients before and after treatment 6 untreated patients 40 treated patients in remission	Samples from 62 control subjects (11 F, 51 M; mean age 36.2 *y*; range 22–72)
Biopsies for cytokine analysis	Samples from 27 CWD patients (1 F, 26 M; mean age 55.3 y; range 41–74) 1 patient before and after treatment 8 untreated patients 18 treated patients in remission	Samples from 42 control subjects (0 F, 42 M; mean age 31.1 y; range 22–48)
Biopsies for histology	Samples from 58 CWD patients (12 F, 24 M; mean age 58.3 y; range 33-84) 20 patients before and after treatment 3 untreated patients 35 treated patients in remission	Samples from 27 control subjects (11 F, 16 M; mean age 46.7 y; range 24-72)

F, female; M, male.

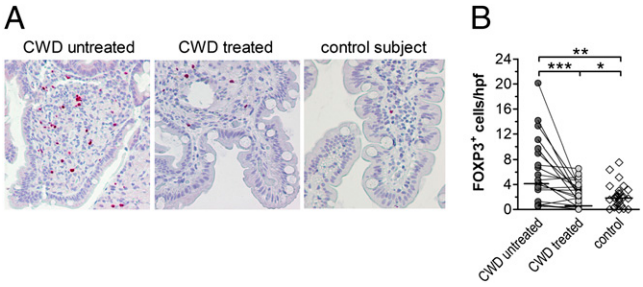

FIGURE 1. T_{reg} in duodenal tissue of CWD patients. Immunohistochemical staining of FOXP3 in duodenal biopsy specimens from CWD patients with untreated disease and treated, that is, after antibiotic therapy as well as from control subjects with no sign of *T. whipplei* infection. *A*, Representative examples of FOXP3 staining (*red*). Original magnification ×400. *B*, Numbers of $FOXP3^+$ cells in samples from untreated and treated CWD patients and from healthy subjects. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, according to Mann–Whitney *U* test.

labeled T_{resp}, and stimulated with anti-CD3/anti-CD28 before the assessment of IFN-γ production of proliferating cells (Fig. 4*A*, 4*B*). T_{reg} from CWD patients suppressed the production of IFN-γ by T_{resp} more effectively than T_{reg} from age-matched healthy controls (Fig. 4*B*). To test whether a suppressive T cell activity abolishes the *T. whipplei*-specific T cell response, we compared the production of IFN-γ of proliferating total $CD4^+$ T cells and T_{reg}-depleted T_{resp} populations (Fig. 4*C*, 4*D*). To this end, CFSE-labeled total $CD4^+$ T cells and T_{reg}-depleted T_{resp} from treated CWD patients or healthy controls were incubated with autologous $CD4^{-/low}$ cells containing APC and were stimulated with *T. whipplei* lysate. The depletion of T_{reg} did not influence the *T. whipplei*-specific IFN-γ production in proliferating $CD4^+$ T cells from CWD patients and control subjects.

Peripheral $CD4^+$ T cells from CWD patients are anergic and hyporesponsive

The impaired *T. whipplei*-specific Th1 response shown earlier (10) might result from T cell anergy induced by the anti-inflammatory cytokine milieu and the increased T_{reg} activity observed in CWD patients described above. To test this hypothesis, we examined the proliferation mediator GITR (32) and the exhaustion marker PD-1 (33) on $CD25^+FOXP3^-$ $CD4^+$ effector T cells and the expression levels of the apoptosis-mediating receptor Fas (CD95) on $CD45RO^+$ $CD4^+$ memory T cells from the peripheral blood of CWD patients before and after therapy and from age-matched healthy controls using seven-color flow cytometry (Supplemental Fig. 3*A*).

Irrespective of their treatment status the percentage of GITR expressing activated effector $CD4^+$ T cells (Fig. 5*A*) was ~2-fold lower in CWD patients than in healthy controls. In contrast, the portion of PD-1 expressing activated effector $CD4^+$ T cells was increased in CWD as compared with healthy controls and dropped upon successful treatment (Fig. 5*B*). Patients with untreated CWD showed also a significantly increased expression of CD95, especially on memory $CD4^+$ T cells (Fig. 5*C*), that decreased to the level of healthy controls after antibiotic treatment.

To confirm anergy of the $CD4^+$ T cells in CWD patients, we determined Th1 and Th17 responses after polyclonal activation of whole blood with the superantigen SEB (Supplemental Fig. 3*B*). As expected by the observed anergic phenotype, $CD4^+$ T cells from patients with untreated CWD showed also a reduced production of IFN-γ (Fig. 5*D*) and of IL-17A (Fig. 5*E*) after stimulation with SEB as compared with healthy controls.

Discussion

The present study provides evidence for a role of T_{reg} in the persistence of *T. whipplei* as it demonstrated an expansion of the T_{reg} population in the duodenal mucosa and an enhanced activity of T_{reg} in the peripheral blood in particular during CWD before onset of treatment.

Up to now, several aspects of the immunology of CWD pointed to an influence of immunoregulation in its pathogenesis. Massive infiltration of the duodenal mucosa with *T. whipplei*-infected macrophages is a hallmark of CWD (4, 5). But despite this impressive presence of the pathogen, only moderate local inflammatory reactions with reduced numbers of $CD4^+$ lamina propria and $CD8^+$ intraepithelial lymphocytes evolve in CWD patients (14). In contrast, CWD patients usually present with elevated values for blood sedimentation rate and serum C-reactive protein (6) and show an activation of peripheral T cells (10, 15). A bias toward enhanced immunoregulatory and Th2 functions and alleviated inflammatory and Th1 reactivity in CWD patients (10–16) as well as the role of T_{reg} during other chronic infections of the gut (19–24) or the periphery (34–36) suggest critical involvement of T_{reg} also in the pathogenesis of CWD. In addition, the etiology of an immune reconstitution inflammatory syndrome during CWD (37) points to an important role of cell-mediated immunoregulation in the course of CWD, too.

Increased numbers of $FOXP3^+$ T_{reg} and their decline after therapy paralleled by a general decrease in the numbers of lamina propria $CD4^+$ and intraepithelial $CD8^+$ lymphocytes (14) in the duodenal mucosa of CWD patients, the tissue with the major pathogen load, support the thesis that T_{reg} affect the pathogenesis of CWD.

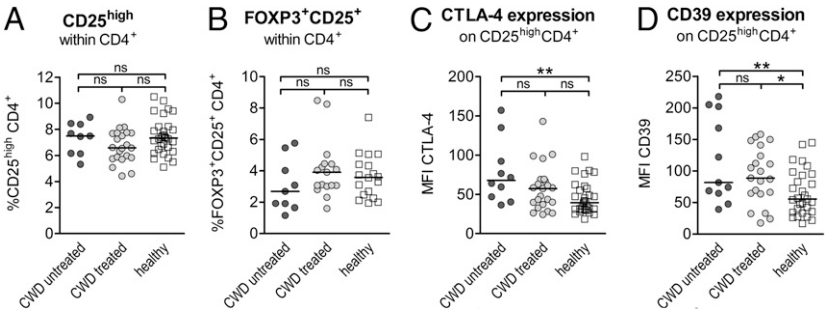

FIGURE 2. Regulatory phenotype of the $CD4^+$ T cells in the peripheral blood of CWD patients. The percentages of $CD25^{high}$ (*A*) and $FOXP3^+CD25^+$ (*B*) cells within the $CD4^+$ T cell population and the expression levels of CTLA-4 (*C*) and CD39 (*D*) on $CD4^+CD25^{high}$ T cells in the peripheral blood from untreated and treated CWD patients and healthy subjects were determined by flow cytometry. *A*, *C*, and *D*, Surface staining of CD4, CD25, CTLA-4, or CD39 performed in whole blood before lysis of erythrocytes. *B,* FOXP3 was stained intranuclearly after lysis of erythrocytes, fixation, and permeabilization. $*p < 0.05$, $**p < 0.01$, according to Mann–Whitney *U* test. MFI, mean fluorescence intensity.

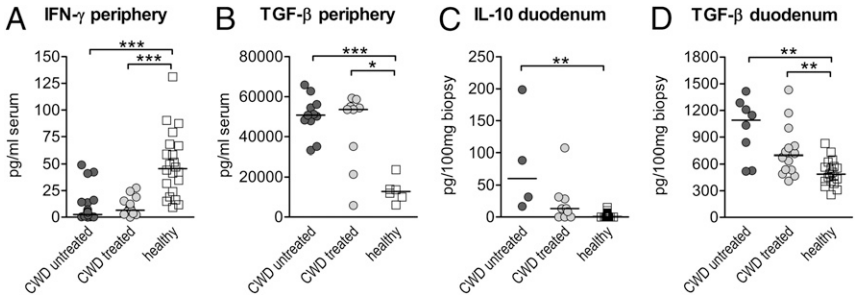

FIGURE 3. Cytokine milieu in the periphery and in duodenal tissue of CWD patients. The concentration of IFN-γ (*A*), TGF-β (*B*, *D*), and IL-10 (*C*) was measured by CBA in serum samples (*A*, *B*) and supernatants of cultured duodenal biopsies (*C*, *D*) from CWD patients before and after treatment and healthy control subjects. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, according to Mann–Whitney *U* test.

Although the percentage of T_{reg} in the peripheral blood was not elevated in CWD patients compared with healthy controls, T_{reg} from CWD patients showed an augmented surface expression of CTLA-4 that mediates inhibitory signals upon CD28 ligation (26, 27). The expression of the ATP-hydrolyzing ectonucleotidase CD39 that abrogates ATP-induced proinflammatory effects (27–29) necessary to suppress IL-17 production (28) was also found to be enhanced in T_{reg} of CWD patients. Thus, altered expression of regulatory effector molecules in peripheral T_{reg} of CWD patients revealed a phenotype with a higher regulatory capacity as compared with T_{reg} from healthy controls. As for the reasons of this enhanced T_{reg} potential, we showed a systemic shift toward a regulatory cytokine milieu. As previously published by us (14), the concentration of the regulatory cytokine IL-10 in the serum and the amount of IL-10 mRNA in duodenal biopsies are increased in CWD patients as compared with healthy controls. In addition, in this paper, we demonstrate that elevated concentrations of IL-10 and TGF-β are locally produced by the duodenal tissue. These surrogate parameters suggested more pronounced activity of T_{reg} from CWD patients that was indeed confirmed in vitro by correspondingly high suppression of IFN-γ expression of active proliferating autologous T cells by peripheral T_{reg} from CWD patients. In accordance with other studies, enhanced expression of CTLA-4 and CD39 on T_{reg} of CWD patients might mediate enhanced suppressive capacity (27–29). However, because a depletion of T_{reg} did not impact *T. whipplei*-specific Th1 reactivity, T_{reg} activation does not seem to be responsible for the absence of *T. whipplei*-specific Th1 reactivity in CWD patients but rather seems to contribute to the control of unspecific inflammatory processes. However, there was no significant difference in the suppression of proliferation of T cells by T_{resp} from CWD patients and healthy subjects in general, probably because of an incubation time of only 4 d that was not optimal to monitor proliferation.

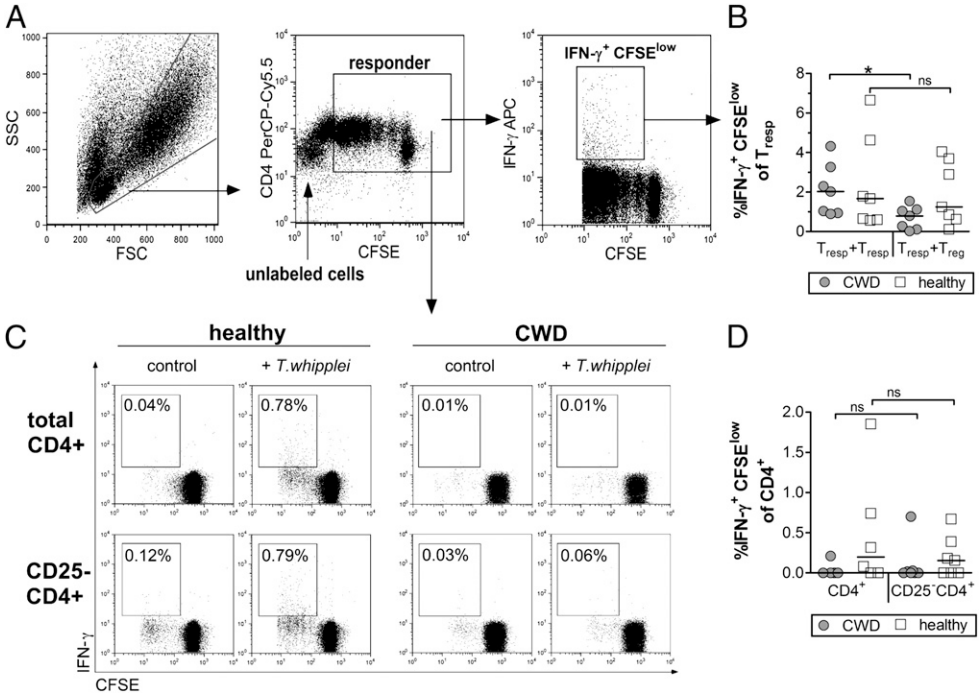

FIGURE 4. Suppression of $CD4^+$ T cell responses by T_{reg} from CWD patients and healthy subjects in vitro. Magnetically sorted $CD4^+$ T cells, $CD25^-$ $CD4^+$ T_{resp}, and $CD25^+CD4^+$ T_{reg} from treated CWD patients and healthy age-matched control subjects were used for in vitro suppression assays. *A* and *B*, To assess the general suppressive activity, CFSE-labeled T_{resp} cocultured either with equal amounts of T_{resp} or T_{reg} were stimulated with anti-CD3/ anti-CD28 Ab. *C* and *D*, To analyze the influence of T_{reg} on *T. whipplei*-specific $CD4^+$ T cell reactivity, CFSE-labeled total $CD4^+$ T cells or T_{reg}-depleted $CD4^+$ T cell populations were stimulated with *T. whipplei* lysate in the presence of autologous $CD4^{-/low}$ cells containing APC. *A*, Live cells were defined by forward light scatter (FSC) and side scatter (of light) (SSC) properties and $CFSE^-$ cells (containing T_{reg} [*B*] or APC [*C*, *D*]) were excluded from the analysis. Only CFSE-labeled cells were further analyzed concerning CFSE dilution and intracellular IFN-γ (*A–D*). *B*, Percentages of proliferating ($CFSE^{low}$) IFN-γ^+ cells within the T_{resp} population. *C* and *D*, Comparison of the *T. whipplei*-specific reactivity of total $CD4^+$ and T_{reg}-depleted $CD4^+$ T cells. *C*, Representative dot plots from a healthy subject and a CWD patient without stimulus (control) or after the addition of *T. whipplei* lysate. *D*, Percentages of proliferating ($CFSE^{low}$) IFN-γ^+ *T. whipplei*-specific cells within the $CD4^+$ population. $*p < 0.05$, according to Mann–Whitney *U* test.

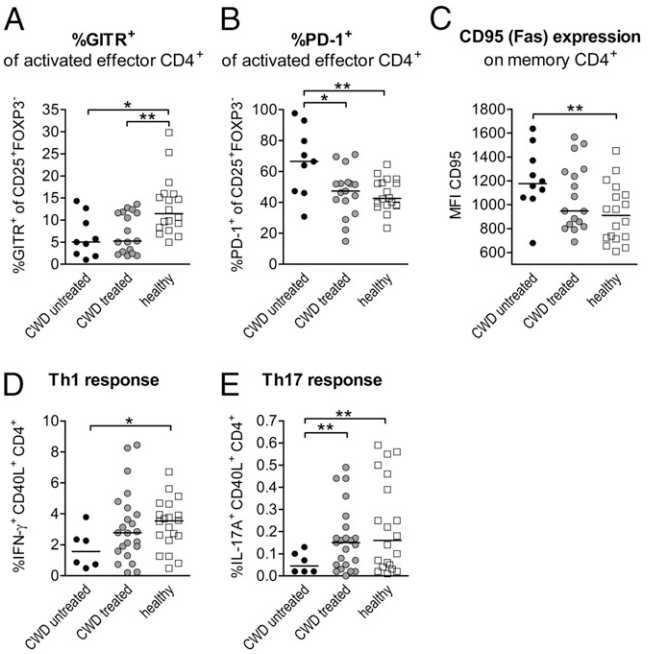

FIGURE 5. Phenotype and responsiveness of peripheral $CD4^+$ T cells in CWD patients. *A–C*, Phenotypic analysis of $CD4^+$ T cells in the periphery from untreated and treated CWD patients and healthy control subjects. Surface staining of CD4, CD45RO, CD25, GITR, PD-1, and CD95 was performed in lysed whole blood. FOXP3 was stained intranuclearly after fixation and permeabilization. The percentages of activated effector $CD4^+$ T cells expressing GITR (*A*) and PD-1 (*B*) as well as the mean fluorescence intensity (MFI) of CD95 on memory $CD4^+$ T cells (*C*) were assessed by seven-color flow cytometry. *D* and *E*, Whole-blood specimen from untreated and treated CWD patients and healthy control subjects were incubated for 6 h with 2 μg/ml SEB. The percentages of $CD40L^+IFN\text{-}\gamma^+$ (*D*) and $CD40L^+IL\text{-}17A^+$ (*E*) within $CD4^+$ T cells were analyzed by four-color flow cytometry. $*p < 0.05$, $**p < 0.01$, according to Mann–Whitney *U* test (*A–D*) and Welch correction for unequal group variances (*E*).

Aside from evidence that natural $FOXP3^+$ T_{reg} mature in the thymus, $FOXP3^+$ T_{reg} can be induced from memory $CD4^+$ T cells in the GALT via dendritic cells in the presence of TGF-β or by lamina propria macrophages (30, 31). Thus, T_{reg} in CWD patients might be generated through Ag presentation by alternatively activated macrophages (14, 38) in close proximity to the site with major pathogen load, the GALT. In return, $FOXP3^+$ T_{reg} have been demonstrated to be potent inducers of alternative macrophage activation (39) that seems to be essential for the persistence of *T. whipplei*. From the intestinal mucosa, following the systemic spread of the pathogen, T_{reg} might disperse to other organ systems.

During CWD, T_{reg} appear to influence the phenotype of $CD4^+$ T cells also indicating enhanced regulatory activity. The percentage of activated effector $CD4^+$ T cells expressing the proliferation mediator GITR was reduced in CWD patients independent of treatment as compared with healthy controls. Because GITR/GITR ligand signaling renders $CD25^-$ T cells resistant to T_{reg}-mediated suppression at the initiation of the immune response (40), reduced GITR expression suggests that T cells in CWD patients are generally more susceptible to suppression and less responsive to proliferative stimuli via GITR ligand (32, 40). By contrast, the exhaustion marker PD-1 (41, 42) was expressed on the majority of activated effector $CD4^+$ T cells in CWD patient before onset of therapy. CWD patients may display an excess of exhausted $CD4^+$ effector T cells because of chronic stimulation as a consequence of persistent infection (27). In patients with untreated CWD, we identified a significantly increased expression of CD95 especially on memory $CD4^+$ T cells that decreases to the level of healthy controls after antibiotic treatment. Therefore, $CD4^+$ T cells from untreated CWD patients are more prone to Fas ligand-mediated apoptosis (43, 44). An insensitivity of Th2 cells to Fas ligand-mediated apoptosis (43) could explain the bias toward Th2 reactivity in CWD (13). Correspondingly, in this paper, we demonstrate that the exhaustion in the T cell compartment of patients with untreated CWD manifests in transiently impaired Th1 and Th17 responses of effector $CD40L^+CD4^+$ T cells to polyclonal stimuli that reflect their anergic and hyporesponsive status before successful antimicrobial treatment. Because of the grouping of treated and untreated CWD patients and the use of CD69 instead of CD40L as activation marker, we were not able to illustrate the transiently impaired Th1 reactivity to polyclonal stimuli previously (10).

In conclusion, we provide evidence that T_{reg} are involved in the pathogenesis of CWD. During untreated disease, T_{reg} can be found in enhanced numbers in the duodenal mucosa and are more activated in the peripheral blood. Although T_{reg} do not seem to specifically abolish Th1 reactivity to *T. whipplei*, the regulatory milieu in CWD might provide immunoprotection against excessive inflammation by rendering T cells more sensitive to regulation, more resistant to stimulation, and more susceptible to apoptosis.

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Disclosures

The authors have no financial conflicts of interest.

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