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CIRCULATING AND MUCOSAL PREDICTORS OF CLINICAL AND ENDOSCOPIC RESPONSE TO VEDOLIZUMAB TREATMENT: RESULTS OF A PHASE IV PROSPECTIVE INTERVENTIONAL TRIAL

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ABSTRACT

Objective. Vedolizumab (VDZ) is a monoclonal antibody directed against α4β7 integrin heterodimer, approved for patients with inflammatory bowel disease (IBD). This study aimed at identifying circulating and mucosal immunologic predictors of response in patients with active ulcerative colitis (UC) and Crohn's disease (CD).

Design. This is an explorative, prospective, phase IV interventional trial (Eudract n. 2015-003270-32). Consecutive CD and UC patients received open-label VDZ at weeks 0, 2, 6 and 14. A week 10 infusion was performed in CD patients. Patients with clinical response at week 14 were maintained with VDZ every 8 weeks up to 54 weeks. At week 0 and 14 peripheral blood was obtained and endoscopy with biopsies was performed. The expression of surface markers, chemokine receptors and α4β7 heterodimer on peripheral blood and lamina propria lymphocytes was assessed by flow cytometry.

Results. 38 IBD patients (20 UC, 18 CD) were included in the study. At week 14, clinical response and remission rates were 87% and 66%, respectively. Endoscopic response rate was 47%. Among week 14 responders, clinical remission rate at week 54 was 69%. No clinical variables were found to predict either clinical or endoscopic outcomes. On the contrary, higher baseline levels of circulating memory CXCR3+CCR6- CD4+ T cells (Th1 cells) were strongly associated with week 14 clinical response (P=0.0001). Reduced baseline levels of lamina propria memory CXCR3-CCR6+ CD4+ T cells (Th17 cells) and CXCR3+CCR6+ CD4+ T cells (Th1/17 cells) were predictive of endoscopic response (P=0.012 and P=0.005 respectively). Circulating levels of Th1 memory T cells predicted clinical remission in IBD patients at week 54.

Conclusions. The results of this exploratory study uncovered a panel of circulating and mucosal immunological predictors of response to vedolizumab treatment. These data provide further insights on the mechanism of action of vedolizumab in IBD patients

INTRODUCTION

In the last years, the pharmacological armamentarium for the treatment of patients with inflammatory bowel diseases (IBD), i.e. Crohn's disease (CD) and ulcerative colitis (UC) has been steadily expanding, as standard immunosuppressants and antibodies against anti-tumour necrosis factor [1-3] have been complemented by a novel generation of monoclonal antibodies, and several molecules are demonstrating positive results in clinical trials [4]. Among these, vedolizumab (VDZ), the first non-antiTNF monoclonal antibody approved for IBD treatment, is emerging as an effective therapy for both CD and UC patients. Vedolizumab is a monoclonal antibody targeting the integrin alfa4beta7 heterodimer [5], a homing receptor predominantly expressed on T lymphocytes, thus preventing its binding to the endothelial ligand mucosal vascular addressin cell adhesion molecule-1 (MAdCAM1). The interaction between integrin alfa4beta7 and MAdCAM1 has been shown to be critical for the homing of both effector and regulatory T lymphocyte in the intestine. Indeed, in murine models of intestinal inflammation, antibody-mediated blockade of α4, β7, the α4β7 heterodimer or its ligand MAdCAM-1 has been shown to prevent the homing of circulating T cells into the inflamed gut, leading to a reduction in colonic T cell density and overall intestinal inflammation [6-8].

Since its approval for the treatment of IBD patients in 2014, VDZ has demonstrated clinical efficacy in IBD patients both naïve or previously exposed to antiTNF blockers [9,10]. Data from real-world cohorts from North America and Europe have reported rates of clinical remission and clinical response ranging between 24%-39% and 49%-64% in CD, and 23%-49% and 43%-57% in UC, respectively [11-18]. Despite these positive results, however, a significant proportion of patients do not respond to vedolizumab treatment, and must resort either to other treatments or to surgical intervention to control disease activity. Mechanisms underlying primary non-response to vedolizumab are still largely unknown, and efforts are being put to identify variables that might predict response to this therapy, as to potentially

optimize patient management. Across multiple cohorts, previous exposure to anti-TNF agents and a higher level of systemic and mucosal inflammation have emerged as negative predictors of response to vedolizumab therapy both in CD and UC patients [19-23].

The identification of one or more biomarkers able to predict clinical efficacy of vedolizumab in patients with both Crohn's disease and ulcerative colitis would be highly relevant for clinical practice, potentially recommending an early use of this drug in patients with favourable predictors of response. In this context, the knowledge of a definite cellular target of vedolizumab, i.e. the α4β7 integrin heterodimer, lead to speculate that immunological profiling of circulating and intestinal lymphocytes could represent a possible resort for therapeutic management. The adoption of total α4β7 integrin expression levels on the whole population of lymphocytes as a potential therapeutic biomarker, however, may be hampered by the observation that this heterodimer is expressed on a wide range of CD4 and CD8 T cells with both proinflammatory and regulatory potential [24].

Here we report the results of an explorative, prospective, monocentric, interventional study (Eudract n. 2015-003270-32) aimed at identifying one or more immunological predictive factor of response to vedolizumab in patients with IBD. The relative frequencies of circulating and gut-infiltrating inflammatory and regulatory T cell subsets at baseline were correlated with short and long-term clinical and endoscopic response to Vedolizumab. Given the pivotal role of CD4+ T helper subsets in modulating pathogenic responses in IBD patients [25], we focused our attention on memory T helper subsets, including Th1 (CXCR3+CCR6-), Th17 (CXCR3-CCR6+), Th1/17 (CXCR3+CCR6+) as well as on regulatory T cells (CD127-CD25+). Multidimensional cytofluorimetric analyses of circulating and mucosal T helper subsets were performed at baseline and post VDZ treatment.

The results of this exploratory study uncovered a panel of circulating and mucosal immunological predictors of response to vedolizumab treatment. These data provide further insights on the mechanism of action of vedolizumab in IBD patients.

MATERIALS AND METHODS

Human Subjects.

Patients with active ulcerative colitis or Crohn's disease for at least 6 months and aged between 18 and 80 years were enrolled in the study. All subjects provided written informed consent to the study.

Key inclusion criteria included: 1. Active inflammatory bowel disease as defined by HBI > 7 at baseline (Crohn's disease patients) or Mayo score > 6 and <12 (ulcerative colitis patients); 2. Baseline SES-CD > 2 (Crohn's disease patients) or Mayo endoscopic subscore >1 (ulcerative colitis patients) at baseline; 3. Inadequate response with, having lost response to, or being intolerant to a TNF blocker or immunomodulator; or having an inadequate response with, being intolerant to, or demonstrating dependence on corticosteroids; 4. Female subjects of childbearing potential were required to have a negative serum pregnancy test (beta-hCG) at screening and a negative urine pregnancy test at baseline, and must have agreed to use acceptable methods of contraception while receiving protocol-specified medication and for 6 months after stopping the medication; 5. Study subjects must have been negative for colorectal cancer or any associated lesions (e.g. dysplasia). 6. Study subjects were required to be eligible according to the tuberculosis (TB) eligibility assessment, screening, and early detection of reactivation rules, including: a. no history of latent or active TB prior to screening; b. no signs or symptoms suggestive of active TB upon medical history and/or physical examination; c. no recent close contact with a person with active TB or, if there has been such contact, will be referred to a physician specializing in TB to undergo additional evaluation and, if warranted, receive appropriate treatment for latent TB prior to or simultaneously with the first administration of vedolizumab. d. having a chest X-ray (posterior-anterior and lateral views), taken within the 3 months prior to the first administration of study agent and read by a qualified radiologist, with no clinically significant

abnormality, or evidence of current active TB or old inactive TB; 7. Subjects were required to be able to adhere to dose and visit schedules.

Key exclusion criteria included: 1. Female patient wither pregnant, nursing, or planning pregnancy (both men and women) within 18 months after screening (i.e., approximately 6 months following last study infusion); 2. Patients who have received rectal therapy with mesalamine or glucocorticoids in the last 14 days; 3: Patients receiving systemic equivalent prednisone dose > 30 mg/day; 4. Patients with serious infections (e.g., active hepatitis, pneumonia, or pyelonephritis) within 4 weeks of screening. Less serious infections (such as acute upper respiratory tract infection [colds] or a simple urinary tract infection) were needed to be resolving or to have resolved prior to enrolling in the study; 5. Patients with opportunistic infections (e.g., herpes zoster [shingles], cytomegalovirus [CMV], Pneumocystis carinii, aspergillosis, histoplasmosis, or mycobacteria other than TB) within 6 months prior to screening; 6. Subjects with a known infection with human immunodeficiency virus (HIV) and/or hepatitis B or hepatitis C; 7. Patients with current signs and symptoms of systemic lupus erythematosus, or severe, progressive, or uncontrolled renal, hepatic, hematologic, endocrine, pulmonary, cardiac, neurologic, or cerebral diseases. 8. Patients carrier of a transplanted organ (with the exception of a corneal transplant > 3 months prior to screening); 9. Patients with actual malignancy or malignancy within 5 years prior to screening, including cervical carcinoma in situ appropriately treated (except for squamous or basal cell carcinoma of the skin that has been treated with no evidence of recurrence); 10. history of lymphoproliferative disease including lymphoma, or signs and symptoms suggestive of possible lymphoproliferative disease, such as lymphadenopathy of unusual size or location (e.g., nodes in the posterior triangle of the neck, infra-clavicular, epitrochlear, or periaortic areas), or splenomegaly.

Concomitant medications: participants could continue to take mesalamine, up to 30 mg of prednisone (or equivalent) per day, or immunosuppressive agents (azathioprine, 6-

mercaptopurine, and methotrexate), provided that patients were receiving these medications for at least 12 weeks and at a stable dosage for at least 4 weeks before baseline. Rectal therapy with mesalamine or glucocorticoids were required to be discontinued 2 weeks before screening. Patients were ineligible if they had received TNF antagonists within 60 days before enrollment or cyclosporine, thalidomide, or any investigational drugs within 30 days before enrollment, or if they had been treated previously with vedolizumab, natalizumab, efalizumab, or rituximab.

Study design.

IBD patients were screened for disease activity, and for inclusion/exclusion criteria. The screening period was approximately two weeks. It covered the time between informed consent, screening for opportunistic infections including tuberculosis, chest X-ray and the first vedolizumab infusion. After screening, eligible patients received open-label vedolizumab infusion (300 mg) over 30 minutes at weeks 0, 2, 6 and 14 after initiation of treatment. An additional infusion at week 10 was performed in CD patients.

Twenty-four hours before administration of vedolizumab at week 0 and at week 14, a 60 mL of peripheral blood were obtained and a sigmoidoscopy or colonoscopy was performed, in which four biopsies were taken from the area most representative of mucosal inflammation. Follow-up biopsies were taken in the same location of baseline biopsies. Patients with clinical response at week 14 were treated with vedolizumab every 8 weeks for one year, while treatment was stopped in patients not responding to vedolizumab treatment at W14. Patients with CD with a Harvey-Bradshaw Index (HBI) [26] of ≤4 were considered to be in remission, those with ≥5 to have active disease. Responders were defined as patients with a decrease of ≥2 points in HBI compared with baseline or patients who achieved remission as defined above. Patients with UC and a total Mayo Score [27] of ≤2 (bleeding 0 and endoscopy ≤1) were considered to be in remission, those with ≥3 to have active disease.

Responders were defined as patients with a decrease in Mayo Score of ≥30% compared with baseline or those who achieved remission as defined above. Endoscopic response was defined as a decline in SES-CD score [28] of at least 50% (CD) or a reduction in Mayo endoscopic subscore of at least 1 point (UC). Schematic diagram of study design is reported in Figure 1.

Human cells isolation.

Peripheral blood mononuclear cells were isolated by density gradient centrifugation using Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden). For the isolation of lamina propria mononuclear cells (LPMCs), fresh intestinal biopsies were collected in calcium- and magnesium-free Hanks' balanced salt solution (HBSS, Euroclone, Italy) and rapidly processed. Biopsy specimens were initially incubated in a HBSS- 1% pen/strep and gentamicin containing dithiothreitol (DTT, 0.16mg/ml, Sigma-Aldrich) for 15 minutes to remove mucus and adherent bacteria. After extensive washing in HBSS, biopsies were incubated for 50 minutes at 37°C with HBSS, 1% Pen/Strep and gentamicin containing 1mM EDTA (Sigma-Aldrich) to remove epithelial cells. After extensive washing in HBSS, biopsies were then digested in RPMI1640 medium (Euroclone, Italy), containing 1mg/ml collagenase D (Roche, Italy) in a 5% CO2 incubator at 37°C for 1 hour. After incubation, lamina propria mononuclear cells (LPMCs) released from the tissue samples were passed through a 70μm cell strainer and washed with complete RPMI 1640 medium (1% pen/strep, non-essential aminoacids and 10% FBS).

Flow cytometry.

Human cells were stained with combinations of directly conjugated antibodies as specified in Supplementary Table 1, all sourced from BD, eBioscience or Biolegend.

Samples were analyzed by a FACSCanto flow cytometer (BD), gated to exclude non-viable

cells on the basis of light scatter. Data were analyzed using FlowJo software (BD).

Statistical analysis.

Continuous variables were described including number of observations, mean, standard deviation (SD), median, ranges (minimum and maximum) and number of missing values. Categorical variables were described including the frequency and percentage of subjects in each category. Comparisons between groups were performed by Mann-Whitney test. Outliers were detected with Grubb's test. Spearman correlation coefficient was calculated in order to detect any significant correlations among all the immunological variables.

To reduce the number of immunological variables, an oblique component analysis was performed. The aim of the procedure is to divide a set of variables into non-overlapping clusters that can be interpreted as essentially one-dimensional. For each cluster, the procedure computes a centroid component maximizing the sum across clusters of the variation accounted for by the cluster components. The cluster scores were obtained by means of the method of centroid components calculating the unweighted averages of the standardized variables [29,30]. For each cluster, the representative was selected as the variable with the minimum 1-R^2 ratio [31].

For each outcome, two multivariable logistic regression models were performed, the first considering the cluster scores and the second considering the cluster representative. Stepwise procedure was used to select variables. Baseline clinical features were included in the stepwise procedure, for both models. Considering the explorative purpose of this analysis an alpha level of 10% was considered. All analyses were performed using SAS Version 9.4.

Study approval

The study was registered (Eudract n. 2015-003270-32). The Institutional Review Board

approved the study (permission number 566_2015 quinquies) and informed consent was obtained from the patients. The study was performed in accordance with Declaration of Helsinki protocols.

RESULTS

Clinical outcomes of vedolizumab therapy.

Between April 2016 and April 2017, 38 IBD patients (20 UC, 18 CD) were enrolled in the study. Patients' clinical variables at baseline are detailed in Table 1.

At week 14, clinical response and clinical remission were observed in 33 out of 38 (87%) and 25 out of 38 (66%) IBD patients, respectively. Endoscopic response at week 14 was documented in 18 out 38 patients (47%. In CD, week 14 clinical response and remission were observed in 16 out of 18 (89%) and 12 out of 18 (67%) patients, respectively, while endoscopic response was observed in 7 out of 18 (39%) patients. In UC, week 14 clinical response and remission were observed in 17 out of 20 (85%) and 14 out of 20 (70%) patients, respectively, while 11 out of 20 (55%) patients achieved endoscopic response (Figure 2). As per protocol, week 14 responders (n=33) continued vedolizumab infusions after week 14. At week 54, clinical remission was achieved in 25 out of 38 (66%) IBD patients (72% in CD and 60% in UC, respectively) of the original cohort (Figure 2). Among w14 responders, clinical remission at week 54 was achieved in 25 out of 33 (76%) IBD patients (81% in CD and 70% in UC, respectively). During maintenance treatment, vedolizumab was stopped in 1 patient because of a side effect and in 5 patients for loss of response.

Baseline clinical features of patients achieving or not week 14 clinical response and remission and endoscopic response following vedolizumab therapy are summarized in Supplementary Tables 2-5.

Baseline immunological profile in IBD patients.

Baseline proportions of circulating and mucosal T cell subsets, as well as percentages of alfa4beta7 expression on distinct T cell subsets, are reported in Figure 3. Differences in baseline distribution of memory T cell subsets were observed when Crohn's disease and ulcerative colitis patients were separately analyzed: specifically, UC patients were characterized by increased levels of circulating memory T cells (total and alfa4beta7^{+}), and by increased percentages of circulating alfa4beta7^{+} memory CXCR3+CCR6- (Th1) and CXCR3+CCR6+ (Th1/17) CD4+ T cells. In contrast, increased proportions of circulating total Th1 and Th1/17 cells were observed in patients with Crohn's disease.

Associations between immunological variables and week 14 clinical outcomes of vedolizumab therapy.

To evaluate if immune-related features might associate with response to vedolizumab treatment, matching peripheral blood and lamina propria mononuclear cells extracted from endoscopic biopsies from the enrolled patients were assessed at baseline using multidimensional flow cytometry, and correlated with clinical and endoscopic response to vedolizumab therapy.

The analysis of baseline circulating and lamina propria T cell subsets in patients either responders or nonresponders to vedolizumab therapy is reported in Figure 4. Notably, higher baseline levels of circulating memory Th1 cells were strongly associated with clinical response in IBD patients (P=0.0001). The same result was confirmed when UC and CD patients were separately analyzed (P=0.004 and P=0.013, respectively). Higher baseline levels of circulating memory Th1/17 cells were associated with clinical response in the whole IBD cohort and in CD patients (P=0.012 and P=0.026 respectively), but not in UC patients (P>0.05). In contrast with these data, the analysis of gut-homing T cells demonstrated that a lower baseline proportion of alfa4beta7^{+} Th1/17 cells were associated with clinical

response in IBD patients (P=0.005). No lamina propria T cell subset was found to be associated with clinical response in either the whole IBD cohort or in CD/UC subgroups. Noteworthy, an increased proportion of memory Th1 cells were found to be associated with week 14 clinical remission both in the whole IBD cohort and when CD and UC patients were separately analyzed ($p<0.05$ for all comparisons) (Figure 5).

Associations between immunological variables and week 14 endoscopic response.

No circulating T cell subsets were found to be associated with endoscopic response in either the whole IBD cohort or in CD or UC patients when separately analyzed. Importantly, reduced baseline proportions of lamina propria memory CXCR3-CCR6+ CD4+ T cells (Th17 cells) and CXCR3+CCR6+ CD4+ T cells (Th1/17 cells) were significantly associated with endoscopic response in the whole IBD cohort (P=0.012 and P=0.005 respectively) (Figure 6). Similar results were found when UC patients were separately analyzed, i.e. lower baseline lamina propria levels of Th17 and Th1/17 cells were significantly associated with endoscopic response after 14 weeks of vedolizumab treatment (P=0.016 and P= 0.012 respectively).

Associations between immunological variables and week 54 clinical remission.

Of note, higher baseline levels of circulating Th1 cells (p=0.008) and lower baseline levels of lamina propria Th17 (p=0.035) and Th1/17 cells (p=0.018) were found to be significantly associated with clinical remission at week 54 in the whole IBD cohort (Figure 7). Increased baseline levels of Th1 cells were associated with week 54 clinical remission in CD patients ($p<0.05$), while reduced baseline levels of lamina propria Th1/17 cells were associated with week 54 clinical remission in UC (p=0.003).

Definition of clusters for immunological variables.

High correlations within circulating lymphocyte subsets and within mucosal lymphocyte subsets were found, while low correlation coefficients were observed across the two subsets (all between -0.33 and 0.45). Therefore, separate clusters were defined for circulating and mucosal subsets. Two clusters were identified for each group (circulating: cluster 1 and 2, and mucosal: cluster 3 and 4) and pertinent cluster scores were calculated. Clusters and the clusters' representatives are depicted in Figure 8.

Multivariable logistic regression models for clinical and endoscopic outcomes.

In order to identify independent predictors of vedolizumab therapy, a multivariable analysis including both clinical and immunological variables was performed. Results of multivariable logistic regression models for clinical variables, cluster scores and cluster representatives on each outcome have been performed.

Notably, cluster 2 score was positively associated with week 14 clinical response (OR=1.69, 90% CI 1.13 – 2.52, p=0.03), while a longer disease duration was associated with a lower likelihood of response (OR=0.91, 90% CI 0.83 – 0.99, p=0.06). Conversely, cluster 4 score was identified as an independent negative predictor endoscopic response (OR=0.88, 90% CI 0.80 – 0.96, p=0.02). The same conclusion was reached for the models considering the clusters' representatives, since the only statistically significant variable detected was the representative of Cluster 4 (mucosal Th17 cells, OR=0.92, 90% CI 0.86 – 0.98, p=0.04).

No immunological variable was found to independently predict clinical remission at week 14 or 54. Higher probability to reach week 54 remission was associated to male sex (OR=0.12, 90% CI 0.03 - 0.44, p=0.007).

Vedolizumab-induced variations in circulating and mucosal lymphocyte subsets.

Circulating and lamina propria lymphocyte subset analyses were performed in all patients after 14 weeks of vedolizumab therapy. As shown in Figure 9, vedolizumab treatment was associated with a disease-specific variation in the proportion of circulating memory Th1 cells, which were respectively reduced and increased in patients with CD and UC ($p=0.03$ and $p<0.001$). Noteworthy, vedolizumab treatment significantly reduced proportions of lamina propria memory Th17 cells in the whole cohort ($p=0.027$) and in UC patients ($p<0.001$). As expected, vedolizumab therapy led to a highly significant and generalized reduction in the proportion of lamina propria alfa4beta7$^+$ CD4$^+$ T cells (Figure 9). Conversely, a significant increase in circulating alfa4beta7$^+$ Th1/17 and Th17 cell percentages was observed only in patients with Crohn's disease ($p<0.01$ and $p=0.03$, respectively) (Figure 10).

Variations of circulating and lamina propria T cell subsets plotted against clinical and endoscopic response and remission are depicted in Figures 11-13. Importantly, patients not achieving week 14 clinical response to vedolizumab therapy were characterized by a greater increase in the proportion of circulating Th1 cells with respect to week 14 responders. No other variations in circulating or lamina propria T cell subset was found to be associated with week 14 clinical response or with week 14 clinical remission. Noteworthy, patients not achieving week 14 endoscopic response were characterized by a greater increase in circulating alfa4beta7 Tregs and Th17 cells with respect to patients achieving this outcome ($p<0.01$ for both) (Figure 7).

Finally, vedolizumab-induced variations on T cell subsets at week 14 were correlated with clinical remission at week 54. Importantly, patients not achieving week 54 clinical remission were characterized by a significantly higher increase in circulating memory Th1 cells at week 14 with respect to week 54 nonremitters ($p<0.01$) (Figure 7).

DISCUSSION

The medical management of inflammatory bowel diseases is undergoing a profound transformation, as novel monoclonal antibodies and several small molecules are progressively entering the market to complement standard immunosuppressants and TNF blockers [4]. Even if these therapeutic innovations are predicted to improve the outcomes of IBD patients, several issues are accompanying the entry of novel compounds on the market, including their real-life effectiveness and safety with respect to existing drugs. Moreover, as results of direct comparative studies between IBD therapies are still lacking, the future availability of multiple compounds advocates the identification of variables or biomarkers able to predict therapeutic outcomes, as to find the best candidate to a given treatment and thus optimize resources.

We here report that baseline immunological profiling of circulating and mucosal T helper lymphocytes, together with the monitoring of their short-term variations, might be useful to predict clinical and endoscopic response to vedolizumab. Of note, immunological cluster scores and cluster representatives, but no clinical variables, were found to independently predict short-term outcomes of vedolizumab therapy at multivariable analysis.

Across several cohorts, clinical variables found to be associated with vedolizumab outcomes in both CD and UC include previous exposure to anti-tumor necrosis factor antibodies and severe disease activity at baseline [19-23]. A scoring system to identify CD patients most likely to respond to vedolizumab, based upon baseline CRP and albumin levels, previous antiTNF exposure, previous surgery and disease phenotype, has been recently proposed and validated, even if its large-scale applicability may be limited by its reduced specificity [32,33]. Although in our study 18% patients were naïve to biologics, they were not more responsive to vedolizumab treatment as compared to not naïve subjects. Moreover, we did not find a higher response to treatment in patients with low disease activity scores and lower PCR levels at baseline (data not shown). A possible explanation for the divergences

between our data and previous reports may reside in the high clinical response observed in our cohort, which is however comparable to other real-world studies [13-15] and the relatively small number of enrolled patients, which could be inadequate to detect weak predictors of response at multivariable analysis. Interestingly, female sex was identified as an independent negative predictor of clinical remission at 1 year, thus confirming previous data emerging from both GEMINI I post hoc analyses [21] and Swedish National Quality Registry for Inflammatory Bowel Disease (SWIBREG) [17]. Gender-related differences strongly influence drug responses [34], and sex-based immunological differences contribute to variations in the incidence of autoimmune diseases and malignancies, susceptibility to infectious diseases and responses to vaccines in males and females [35]. Thus, even if further data will be necessary to confirm these findings, it is biologically plausible that sex could also exert an influence over the activity of a drug that modulates the function of immune system, such as vedolizumab. In this regard, our data demonstrate that increased levels of circulating CXCR3+ T helper cells are associated with clinical response and remission following vedolizumab therapy. Moreover, short-term variations of this cell subset are associated with week 14 clinical response and week 14 and 54 clinical remission, as vedolizumab non-responders exhibit a greater increase of circulating CXCR3+ T helpers with respect to responders. Taken together, these data suggest that CXCR3 might represent a biological target of vedolizumab activity. CXCR3 is a chemokine receptor highly upregulated on IFN-γ producing effector and memory Th1 cells upon antigen-dependent interaction with dendritic cells, and is therefore accepted as a marker for Th1 lineage in humans [36]. The expression of its ligands CXCL9 (also known as MIG, monokine induced by gamma-interferon), CXCL10 (IP-10, interferon-induced protein of 10kDa), and CXCL11 (I-TAC, interferon inducible T cell alpha chemoattractant) is also increased during immune responses 7,11,12 rev and is dependent on IFN-γ production (40-42). Experimental evidences suggest that CXCR3 is critically involved in the migration of lymphocytes in the

gut during intestinal inflammation, as CXCR3-deficient T helpers cells lose their colitogenic potential during adaptive transfer colitis in mice [37]. Of note, a monoclonal antibody targeting CXCL10 (BMS-936557) has demonstrated potential efficacy in patients with active ulcerative colitis, even if the clinical trial failed to achieve the primary endpoint possibly due to the administration of a suboptimal drug dose [38]. Since vedolizumab response has been associated with a downregulation of mucosal gene expression of *CXCL 9/10/11* [39], it could be speculated that interference with this pathway could represent a complementary mechanism of action of vedolizumab in IBD, given that in our cohort clinical outcomes were associated with CXCR3 expression on total memory CD4+ T cells, rather than on the a4b7+ subpopulation.

Our data demonstrate that endoscopic response to vedolizumab is associated with reduced frequency of CD4+ T cells expressing CCR6 in the lamina propria. CCR6 is a chemokine receptor directly induced by the Th17-related transcription factor RORC, and is required for recruitment of Th17 cells in the inflamed gut upon binding with its cognate ligand CCL20 (also known as MIP3alpha – Macrophage Inflammatory Protein 3). CCR6 is expressed on virtually all IL-17 producing memory cells and is therefore the best available marker for human Th17 cells [40]. The finding of increased levels of Th17 cells, a T cell population with documented pathogenic activity during IBD, in endoscopic non-responders to vedolizumab therapy, might lead to speculate that this cell subset is particularly refractory to biological activity of this drug. Indeed, it has been recently reported that IL-17-producing cells are particularly enriched in a population of resident memory T cells (T_{RM}), a cell population with memory function and long-term persistence in mucosal tissue, with low recirculation capability [41].

We [42-45] and others [46, 47] have reported that CCR6+ Th17 cells manifest an intrinsic plasticity, and might coexpress CXCR3 upon the exposure to IL23 and IL12. These CXC3R+CCR6+ T cells, also known as Th1/17 cells, coproduce IFN-γ and IL17, and are

highly pathogenic towards intestinal epithelium [44]. The association between baseline frequencies of circulating Th1/17 and both clinical and endoscopic outcomes of vedolizumab therapy, is therefore of particular interest, together with the demonstration that their baseline levels independent predict vedolizumab response.

Our data are apparently in contrast with a recent report [48], where no major differences were observed in absolute number, frequency, phenotype, TCR repertoire of mucosal and peripheral CD4, CD8, central and effector memory between remitters and nonremitters to vedolizumab therapy. A possible explanation for these discrepancies may reside in differences in a different depth of phenotypical analysis of T cells, as we aimed at studying T helper subsets rather than the whole CD4 or CD8 mucosal and peripheral T cell populations.

Previous works indicated that alfa4beta7 expression by circulating subsets (B, NK, T and CD4 terminal effector memory) was higher in VDZ responders than in not-responders (Boden DSS 2018). Similarly, a progressive decline of alfa4beta7 expression was reported on systemic Th1, Th2, Th17 (CD) and Th17 (UC) cells and in mucosal T helper subsets in remitters [49]. We were unable to replicate these findings, even if we could demonstrate that vedolizumab therapy led to a progressive depletion of alfa4beta7-positive cells in the lamina propria, both in UC and CD. Differences in outcome definition (clinical vs. endoscopic), study design (retrospective vs. prospective), and technical differences (fresh vs. cryopreserved material), may account for these discrepancies.

In conclusion, the results of this exploratory study uncovered a panel of circulating and mucosal immunological predictors of response to vedolizumab treatment. These data provide further insights on the mechanism of action of vedolizumab in IBD patients.

TABLES

Table 1. Baseline clinical characteristics of the enrolled patients with Crohn's disease.

Clinical parameter	**Ulcerative colitis (n=20)**	**Crohn's disease (n=18)**
Male/Female, n	12/8	14/4
Age at diagnosis, mean ± SD (years)	31 ±14	25 ±9
Age at enrolment, mean ± SD (years)	47 ±15	39 ±17
Disease duration, mean ± SD (years)	9 ±7	16 ±10
Smoking status, yes/no	1/19	4/14
Disease location* (ulcerative colitis), n*		
E1 (proctitis)	0	-
E2 (left sided)	10	-
E3 (extensive)	10	-
Disease location* (Crohn's disease), n*		
L1 (ileal)		8
L2 (colonic)		1
L3 (ileocolonic)		8
L4 (upper disease)		1
Disease behaviour* (CD), n*		
B1 (non-stricturing, non-penetrating)	-	8
B2 (stricturing)	-	6
B3 (penetrating)	-	4
Concomitant therapy at enrolment		
mesalamine, n°	20	-
thiopurines, n°	-	1
corticosteroids, n°	5	-
Previous antiTNF treatment, n°	15	16
more than 1 antiTNF, n°	6	11
Mayo score at baseline, mean ± SD	9 ± 2	-
HBI at baseline (mean±SD)	-	7 ±4

*according to the Montreal classification [11].

UC: ulcerative colitis; CD: Crohn disease; SD: standard deviation; HBI: Harvey Bradshow Index

Supplementary Table 1. Combination of directly conjugated antibodies for human cells staining

- Anti-human CD4 APC-Cy7 (clone A161A1)
- Biotin anti-human CD127 (IL-7Ra) (clone MB15-18C9)
- Anti-Biotin VioGreen (Clone REA746)
- Anti-human CD25 Pacific Blue (clone M-A251)
- Anti-human CD196 (CCR6) PE/Cy7 (clone G034E3)
- Anti-human CD183 (CXCR3) PE (clone G025H7)
- Anti-human CD195 (CCR5) FITC (clone J418F1)
- Anti-human CD49d APC (clone 9F10)
- Anti-human Integrin β7 PE/Cy5 (clone FIB504)

Supplementary Table 2. Week 14 clinical response

Clinical parameter	**No (n=5)**	**Yes (n=33)**	**Overall (n=38)**
Disease type, n (%)			
UC	3(60)	17 (52)	20 (53)
CD	2 (40)	16 (49)	18 (47)
Male/female, n	1/4	25/8	26/12
Age at diagnosis, mean ± SD (years)	26±7	32±14	31±13
Age at enrolment, mean ± SD (years)	47±12	43 ±14	43 ±14
Disease duration, mean ± SD (years)	21±18	11±7	12±9
Smoking status, yes/no/ex	0/5	May-25	May-30
Disease location* (ulcerative colitis),n*			
E1 (proctitis)	-	-	0
E2 (left sided)	0	10	10
E3 (extensive)	3	7	10
Disease location* (Crohn's disease),n*			
L1 (ileal)	0	8	8
L2 (colonic)	0	1	1
L3 (ileocolonic)	4	4	8
L4 (upper disease)	0	1	1
Disease behaviour* (CD), n*			
B1 (non-stricturing, non-penetrating)	0	8	8
B2 (stricturing)	1	5	6
B3 (penetrating)	1	3	4
Concomitant therapy at enrolment			
mesalamine, n°	3	17	20
thiopurines, n°	0	1	1
corticosteroids, n°	1	4	5
Previous antiTNF treatment, n°	4	27	31
more than 1 antiTNF, n°	2	15	17
Mayo score at baseline, mean ± SD	10±3	9±2	9±2
HBI at baseline (mean±SD)	8±1	7±4	7±4

*according to the Montreal classification [11]

Supplementary Table 3. Week 14 clinical remission

Clinical parameter	**No (n=12)**	**Yes (n=26)**	**Overall (n=38)**
Disease type, n (%)			
UC	6(50)	14 (54)	20(53)
CD	6 (50)	12 (46)	18(47)
Male/female, n	6/6	20/6	26/12
Age at diagnosis, mean ± SD (years)	26±9	33±15	31±13
Age at enrolment, mean ± SD (years)	42±12	44 ±15	43 ±14
Disease duration, mean ± SD (years)	15±12	11±8	12±9
Smoking status, yes/no/ex	1/10	4/20	5/30
Disease location* (ulcerative colitis),n*			
E1 (proctitis)	-	-	0
E2 (left sided)	2	8	10
E3 (extensive)	4	6	10
Disease location* (Crohn's disease),n*			
L1 (ileal)	1	7	8
L2 (colonic)	0	1	1
L3 (ileocolonic)	6	2	8
L4 (upper disease)	0	1	1
Disease behaviour* (CD), n*			
B1 (non-stricturing, non penetrating)	2	6	8
B2 (stricturing)	3	3	6
B3 (penetrating)	2	2	4
Concomitant therapy at enrolment			
mesalamine, n°	6	1	20
thiopurines, n°	0	1	1
corticosteroids, n°	1	4	5
Previous antiTNF treatment, n°	11	20	31
more than 1 antiTNF, n°	7	10	17
Mayo score at baseline, mean ± SD	10±2	9±2	9±2
HBI at baseline (mean±SD)	8±4	6±3	7±4

*according to the Montreal classification [11]. SD: standard deviation.

Supplementary Table 4. Week 14 endoscopic remission.

Clinical parameter	**No (n=20)**	**Yes (n=18)**	**Overall (n=38)**
Disease type, n (%)			
UC	9(45)	11 (61)	20 (53)
CD	6 11(55)	7 (39)	18 (47)
Male/female, n	12/8	14/4	26/12
Age at diagnosis, mean ± SD (years)	27±11	35±14	31±13
Age at enrolment, mean ± SD (years)	43±14	44 ±15	43 ±14
Disease duration, mean ± SD (years)	15±11	9±6	12±9
Smoking status, yes/no/ex	3/14	2/16	5/30
Disease location* (ulcerative colitis),n*			
E1 (proctitis)	-	-	-
E2 (left sided)	4	6	10
E3 (extensive)	5	5	10
Disease location* (Crohn's disease),n*			
L1 (ileal)	4	4	8
L2 (colonic)	0	1	1
L3 (ileocolonic)	6	2	8
L4 (upper disease)	1	0	1
Disease behaviour* (CD), n*			
B1 (non-stricturing, non-penetrating)	3	5	8
B2 (stricturing)	5	1	6
B3 (penetrating)	3	1	4
Concomitant therapy at enrolment			
mesalamine, n°	99	11	20
thiopurines, n°	1	0	1
corticosteroids, n°	2	3	5
Previous antiTNF treatment, n°	18	13	31
more than 1 antiTNF, n°	10	7	17
Mayo score at baseline, mean ± SD	10±2	9±2	9±2
HBI at baseline (mean±SD)	7±4	7±4	7±4

*according to the Montreal classification [11]. SD: standard deviation.

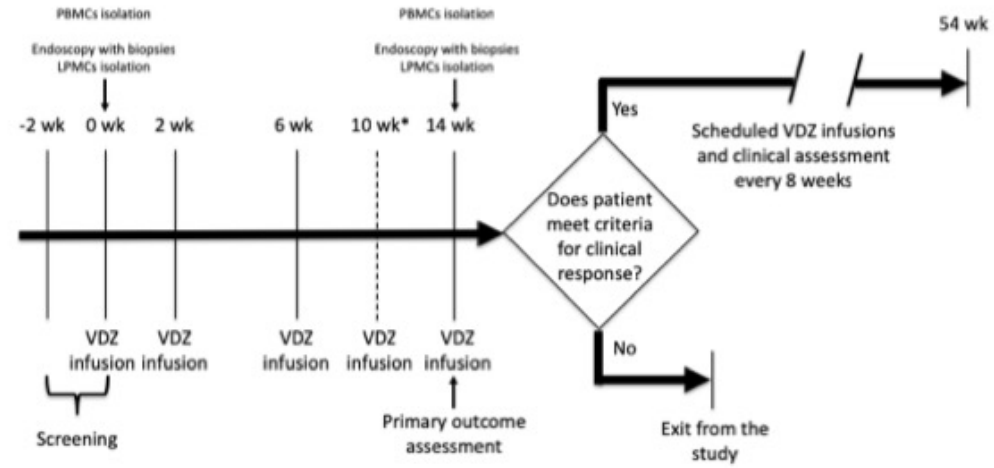
Supplementary Table 5. Week 54 clinical remission.

Clinical parameter	**No (n=13)**	**Yes (n=25)**	**Overall (n=38)**
Disease type, n (%)			
UC	8(62)	12 (48)	20 (53)
CD	6 5(39)	13 (52)	18 (48)
Male/female, n	5/8	21/4	26/12
Age at diagnosis, mean ± SD (years)	27±13	33±14	31±13
Age at enrolment, mean ± SD (years)	44±13	43 ±15	43 ±14
Disease duration, mean ± SD (years)	15±13	11±7	12±9
Smoking status, yes/no/ex	1/10	4/20	5/30
Disease location* (ulcerative colitis),n*			
E1 (proctitis)	-	-	0
E2 (left sided)	3	7	10
E3 (extensive)	5	5	10
Disease location* (Crohn's disease),n*			
L1 (ileal)	3	5	8
L2 (colonic)	-	1	1
L3 (ileocolonic)	4	4	8
L4 (upper disease)	0	1	1
Disease behaviour* (CD), n*			
B1 (non-stricturing, non-penetrating)	2	6	8
B2 (stricturing)	3	3	6
B3 (penetrating)	2	2	4
Concomitant therapy at enrolment			
mesalamine, n°	8	12	20
thiopurines, n°	1	0	1
corticosteroids, n°	2	3	5
Previous antiTNF treatment, n°	12	19	31
more than 1 antiTNF, n°	8	9	17
Mayo score at baseline, mean ± SD	11±2	8±2	9±2
HBI at baseline (mean±SD)	9±5	6±2	7±4

*according to the Montreal classification [11]. SD: standard deviation.

FIGURES

Figure 1: Schematic diagram of study design



* An additional VDZ infusion at week 10 was performed in the CD patients subgroup

Figure 1

Figure 2: Proportion of patients subdivided according to IBD, CD and UC achieving week 14 clinical response (A), clinical remission (B) and endoscopic remission (C) and week 54 clinical remission (D)

IBD: total inflammatory bowel disease
CD: crohn's disease
UC: ulcerative colitis

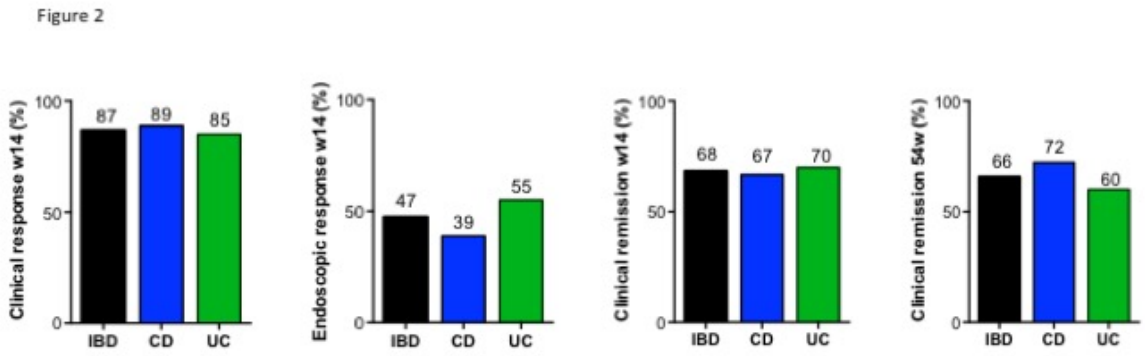


Figure 3: Baseline proportions (A) and percentages of alfa4beta7 expression (B) of circulating and mucosal T cell subsets in IBD, Crohn Disease and Ulcerative colitis patients.

IBD: inflammatory bowel disease

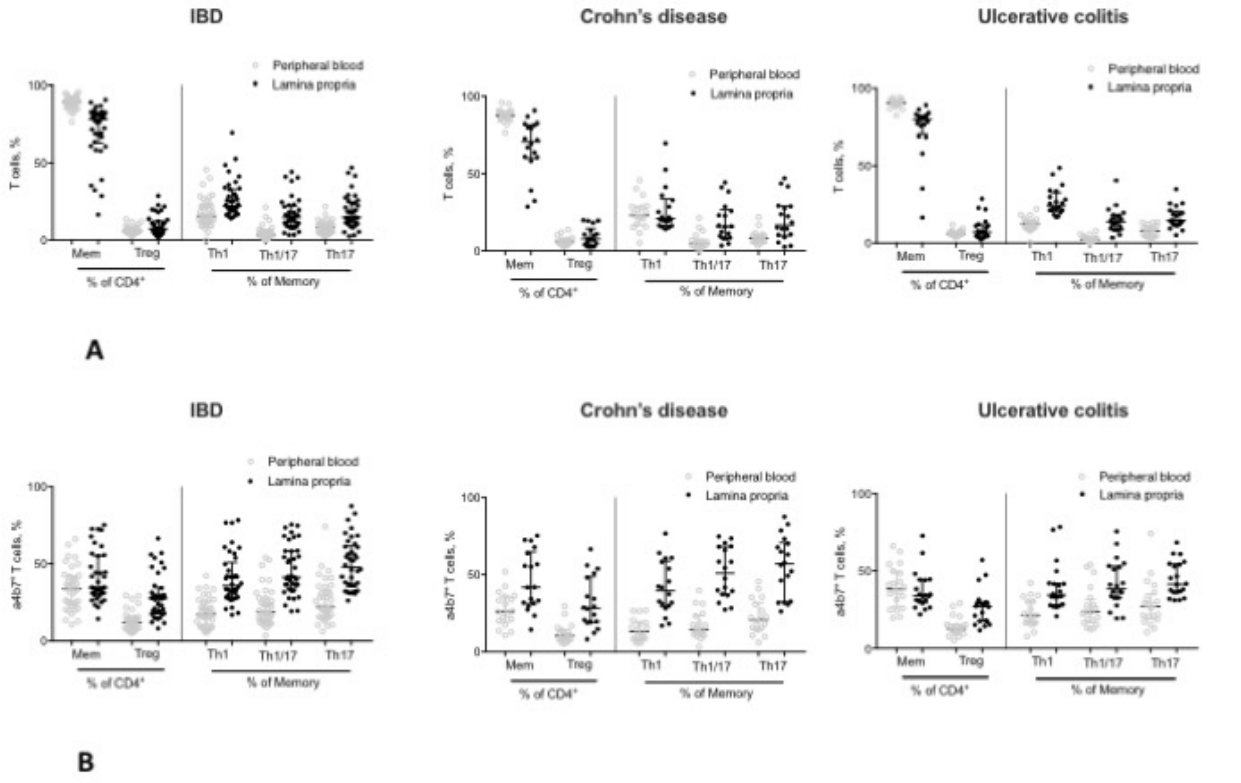


Figure 3

Figure 4: Baseline proportion and percentage of circulating (A and B, respectively) and lamina propria (C and D, respectively) T cell subsets in patients either responders or nonresponders to vedolizumab therapy at week 14.

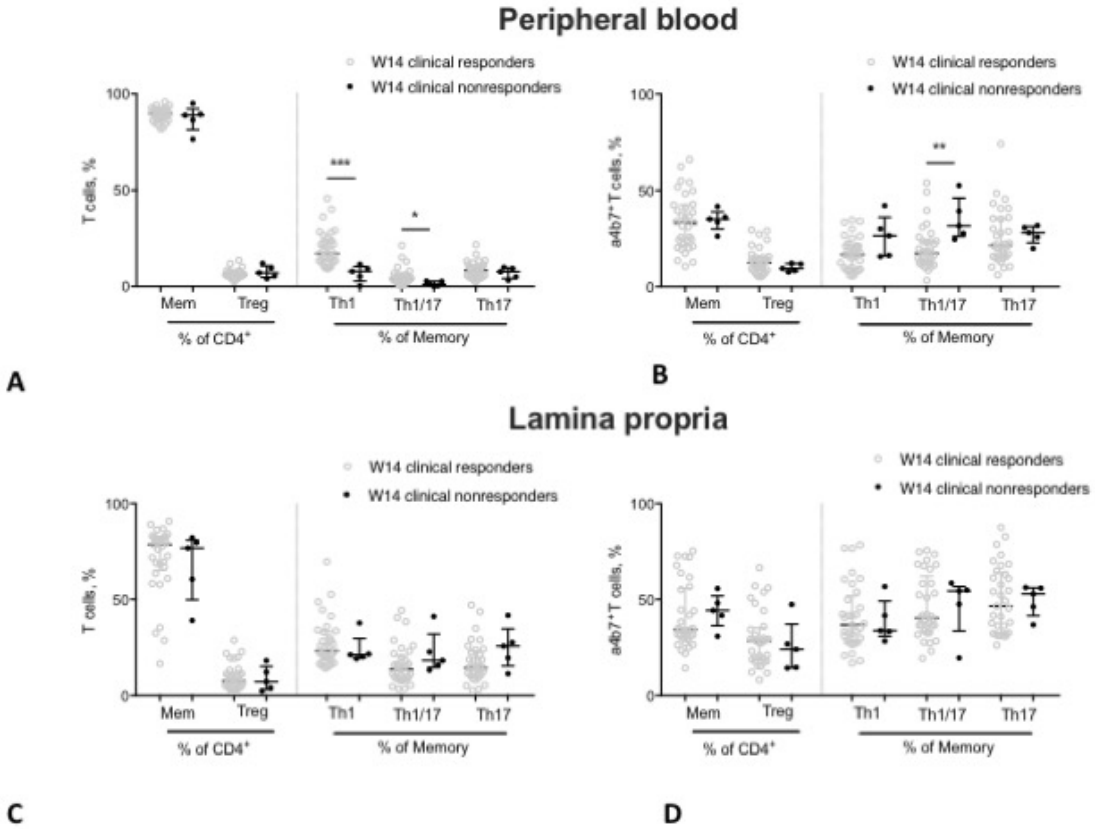


Figure 4

Figure 5: Baseline proportion and percentage of circulating (A and B, respectively) and lamina propria (C and D, respectively) T cell subsets in patients either remitters or nonremitters to vedolizumab therapy at week 14.

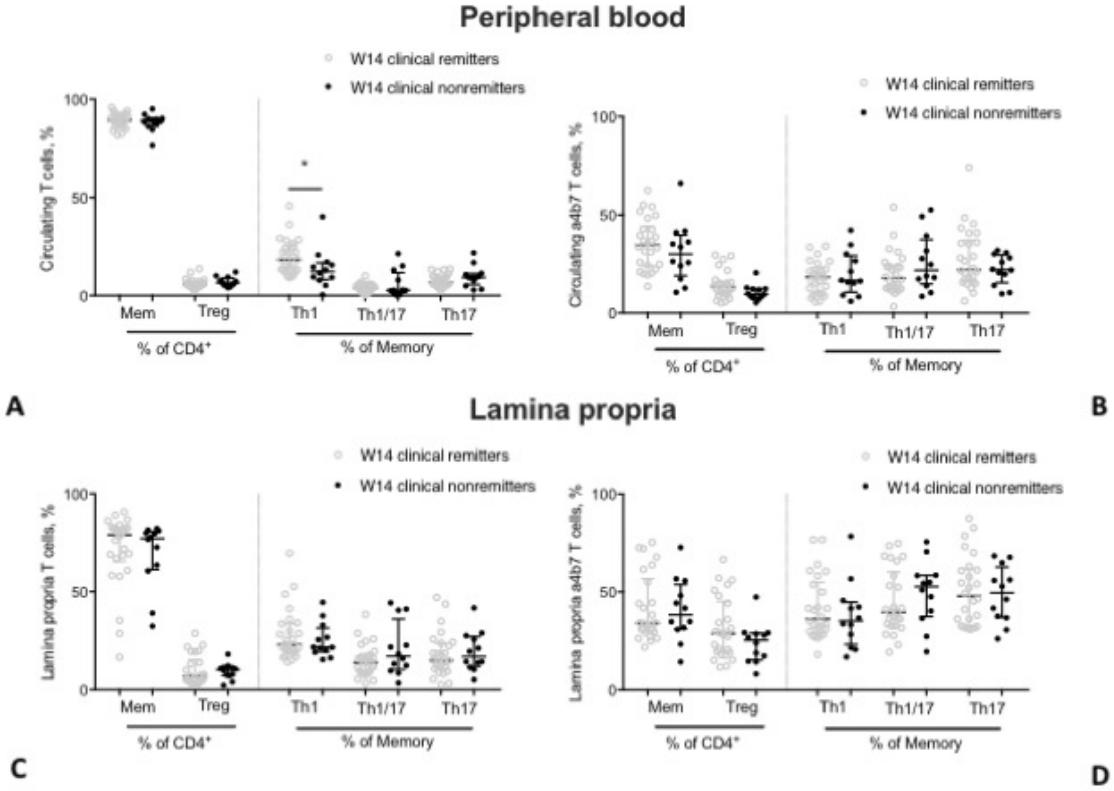


Figure 5

Figure 6: Baseline proportion and percentage of circulating (A and B, respectively) and lamina propria (C and D, respectively) T cell subsets in patients either endoscopic responders or nonresponder to vedolizumab therapy at week 14.

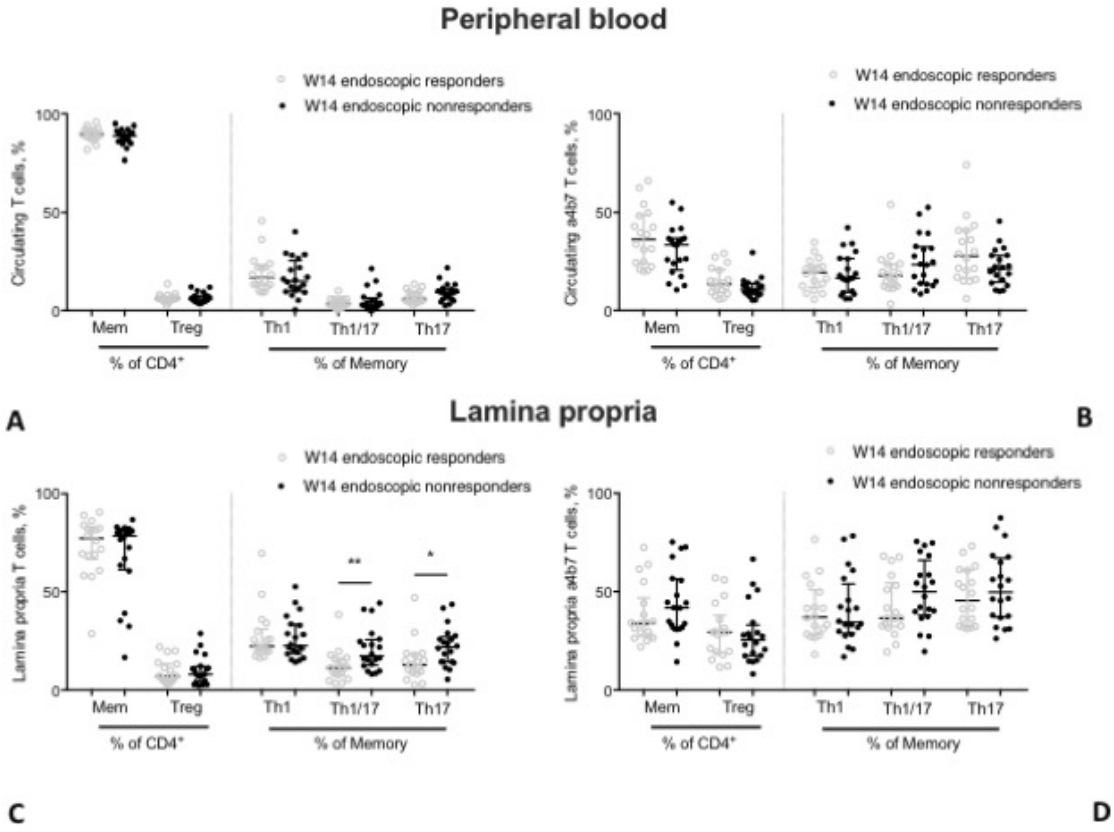


Figure 6

Figure 7: Baseline proportion and percentage of circulating (A and B, respectively) and lamina propria (C and D, respectively) T cell subsets in patients either remitters or nonremitters to vedolizumab therapy at week 54.

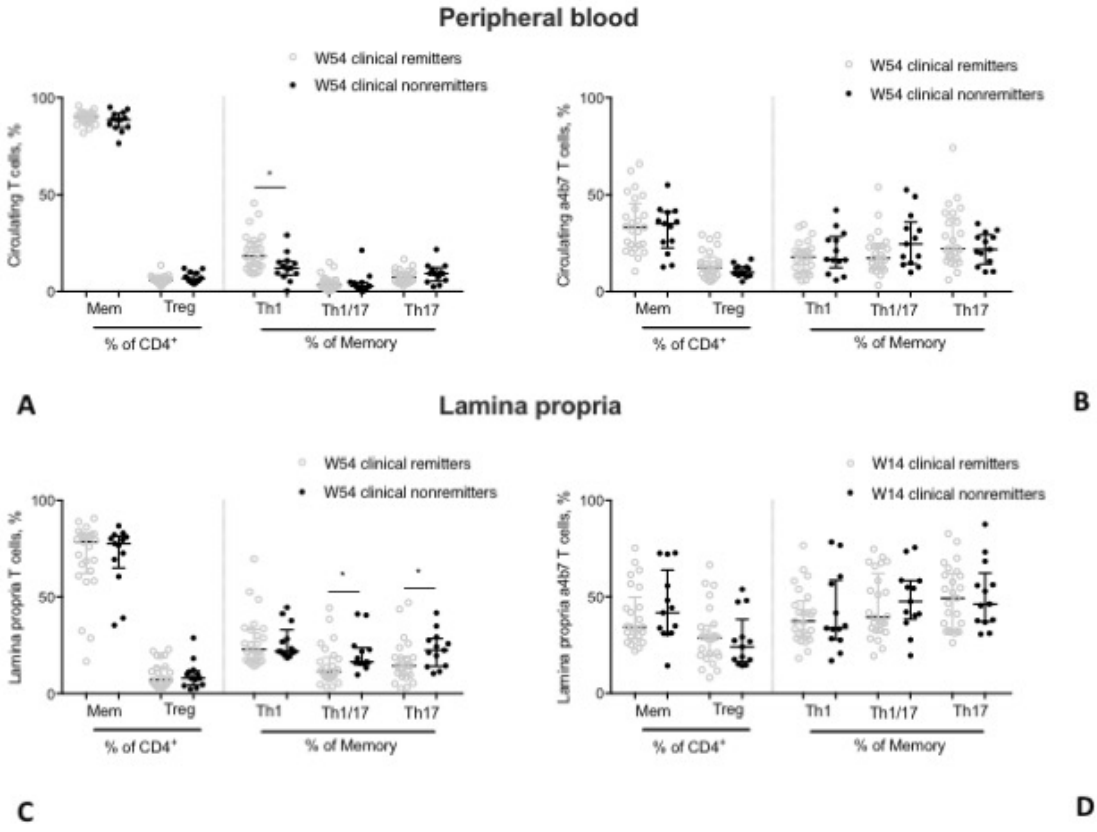


Figure 7

Figure 8: Description of clusters and the clusters' representatives.

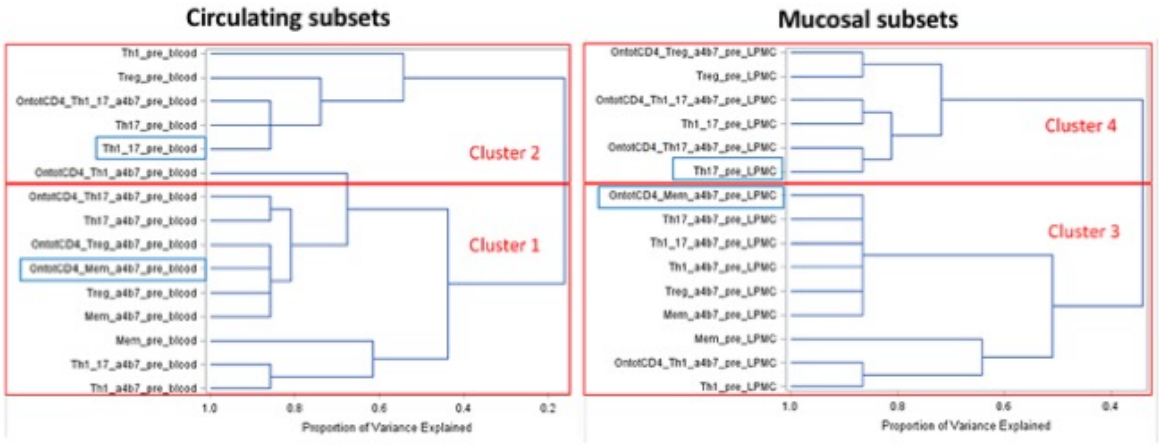


Figure 8

Figure 9: Vedolizumab-induced variations in proportion of circulating (A) and lamina propria (B) T cell subsets between week0 and week14. Data are shown in IBD, Crohn Disease and Ulcerative Colitis patients respectively.

IBD: inflammatory bowel disease

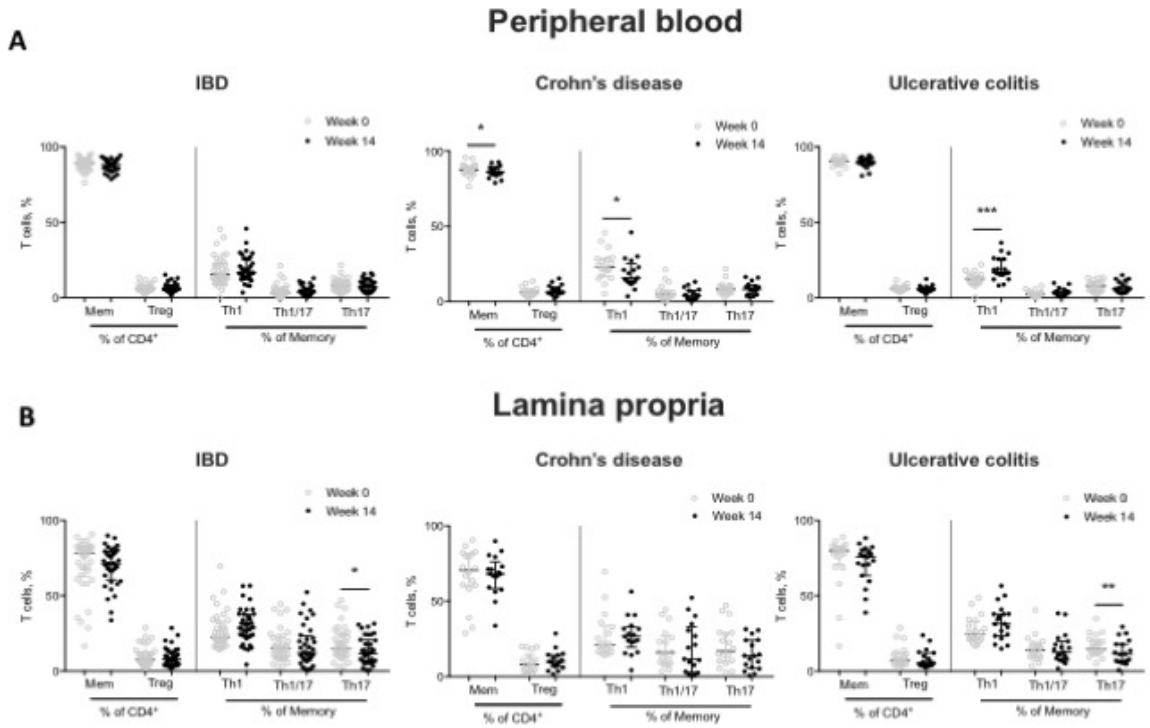


Figure 9

Figure 10: Vedolizumab-induced variations in percentage of circulating (A) and lamina propria (B) T cell subsets between week0 and week14. Data are shown in IBD, Crohn Disease and Ulcerative Colitis patients respectively.

IBD: inflammatory bowel disease

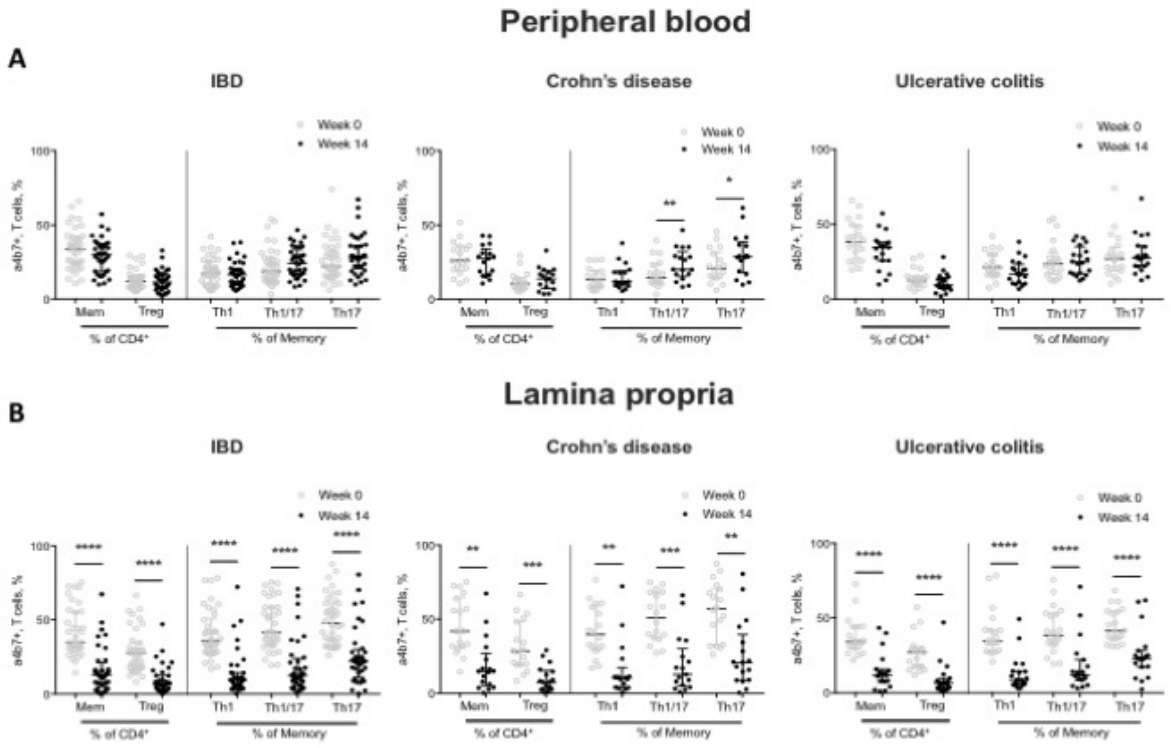


Figure 10

Figure 11: Heatmap showing variations of circulating and lamina propria T cell subsets against week14 clinical response.

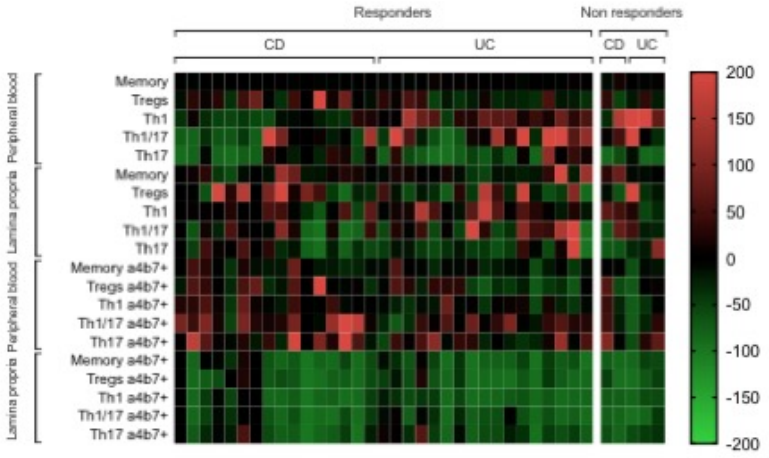


Figure 11

Figure 12: Heatmap showing variations of circulating and lamina propria T cell subsets against week14 endoscopic response.

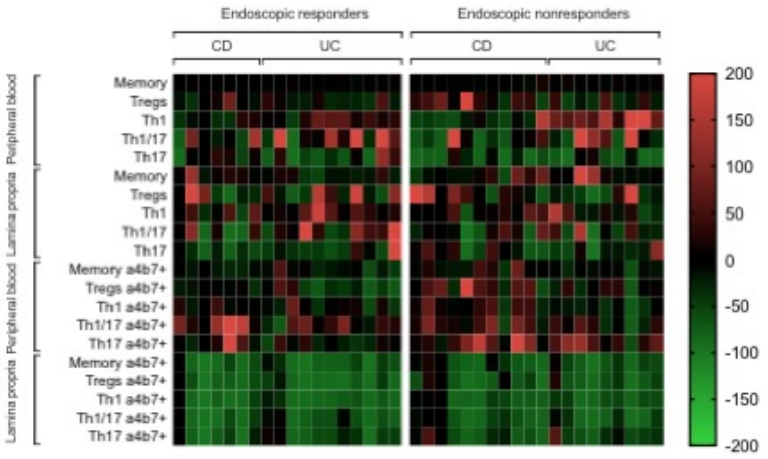


Figure 12

Figure 13: Heatmap showing variations of circulating and lamina propria T cell subsets against week54 clinical remission.

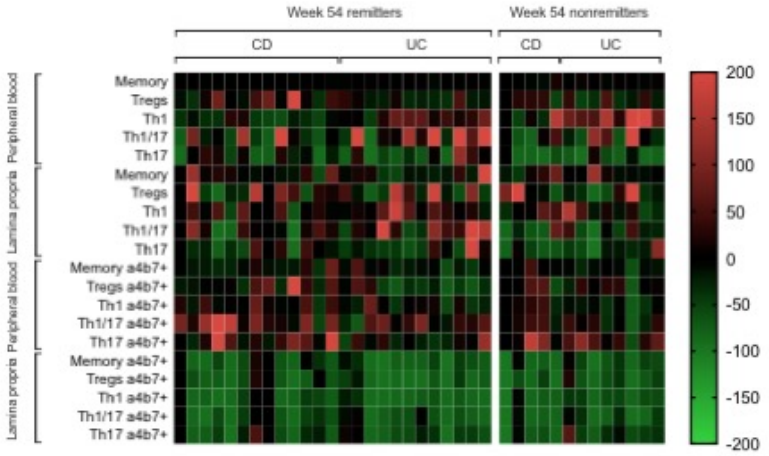


Figure 13

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