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# Multimodal Profiling of Peripheral Blood Identifies Proliferating Circulating Effector CD4$^+$ T Cells as Predictors for Response to Integrin $\alpha4\beta7$–Blocking Therapy in Inflammatory Bowel Disease

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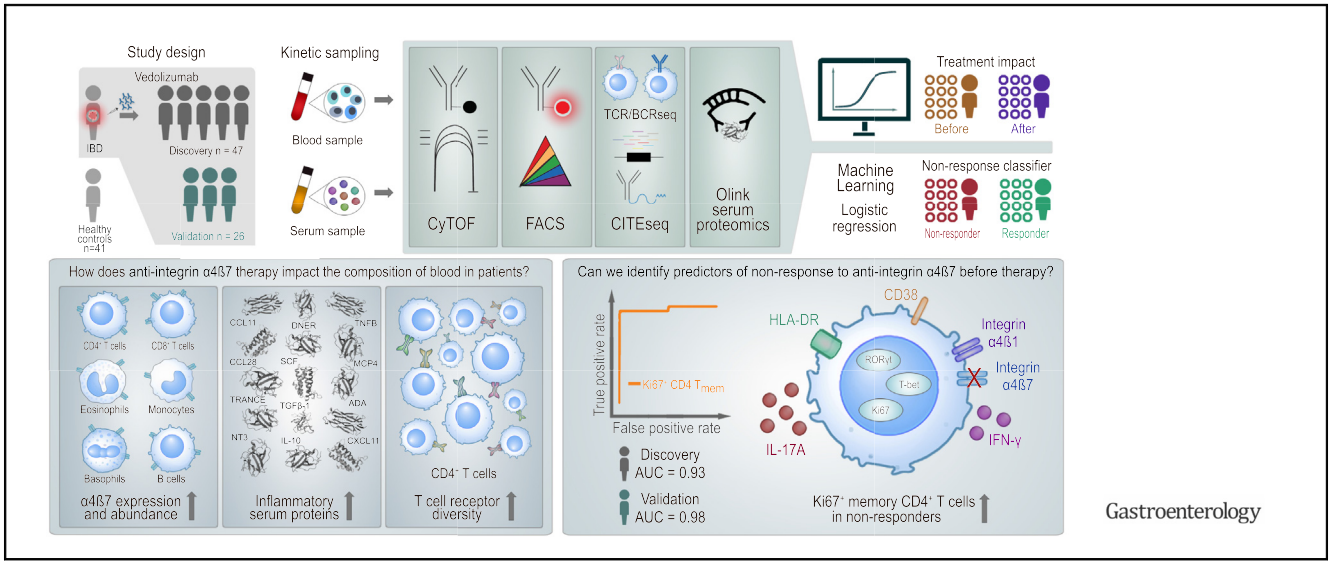


**BACKGROUND & AIMS:** Despite the success of biological therapies in treating inflammatory bowel disease, managing patients remains challenging due to the absence of reliable predictors of therapy response. **METHODS:** In this study, we prospectively sampled 2 cohorts of patients with inflammatory bowel disease receiving the anti-integrin $\alpha4\beta7$ antibody vedolizumab. Samples were subjected to mass cytometry; single-cell RNA sequencing; single-cell B and T cell receptor sequencing (BCR/TCR-seq); serum proteomics; and multiparametric flow cytometry to comprehensively assess vedolizumab-induced immunologic changes in the peripheral blood and their potential associations with treatment response. **RESULTS:** Vedolizumab treatment led to substantial alterations in the abundance of circulating immune cell

lineages and modified the T-cell receptor diversity of gut-homing CD4$^+$ memory T cells. Through integration of multimodal parameters and machine learning, we identified a significant increase in proliferating CD4$^+$ memory T cells among nonresponders before treatment compared with responders. This predictive T-cell signature demonstrated an activated T-helper 1/T-helper 17 cell phenotype and exhibited elevated levels of integrin $\alpha4\beta1$, potentially making these cells less susceptible to direct targeting by vedolizumab. **CONCLUSIONS:** These findings provide a reliable predictive classifier with significant implications for personalized inflammatory bowel disease management.

*Keywords:* Inflammatory Bowel Disease; Cell Migration and Homing; Integrin $\alpha4\beta7$; CD4$^+$ Memory T Cells; Vedolizumab; Single-Cell Profiling; Machine Learning; Therapy Response.



Recent therapies for inflammatory bowel disease (IBD) target pathogenic cytokine pathways and immune cell recruitment to the gut.[1–4] Vedolizumab, a monoclonal antibody, selectively inhibits lymphocyte homing to the intestine by disrupting interactions between mucosal vascular addressin cell adhesion molecule–1 (MAdCAM-1) on gut endothelial cells and integrin $\alpha4\beta7$ on circulating leukocytes.[5–8] Vedolizumab induces and maintains clinical remission in patients with Crohn's disease (CD) and ulcerative colitis (UC).[9–11] It is believed to act on gut-homing T-cell subsets, but recent reports have indicated effects on other cell types known to express integrin $\alpha4\beta7$, such as monocytes, eosinophils, and plasmablasts.[11–13] Integrin $\alpha4\beta7$ blockade increases total numbers of peripheral blood mononuclear cells (PBMCs) and circulating memory T cells, suggesting that it sequesters gut-homing cells in the blood, thereby reducing their numbers in the mucosa and gut-associated lymphoid tissue.[12,14–17]

Despite this knowledge, only a fraction of patients respond to vedolizumab or experience full remission, underscoring the unmet need for a more comprehensive understanding of its mode of action and the factors and mechanisms behind treatment failure.[9,10,18,19] In addition, identifying biomarkers or correlates of therapy failure could help to deploy specific biological therapies to patients with IBD who are more likely to respond.[19–21] There have been attempts to identify potential predictors of therapy response to vedolizumab based on (para-)clinical parameters, such as C-reactive protein, interleukin (IL) 6/8, and integrin $\alpha4\beta7$ expression on circulating and lamina propria T cells, the composition of the fecal microbiota, and cellular and transcriptomic cues in the intestinal mucosa.[22–30] However, none of these biomarkers are clinically established, and the immunologic correlates of anti-integrin treatment responsiveness, especially in peripheral blood, remain undefined.

In this prospective cohort study, we employed multimodal profiling to systematically evaluate the circulating immune landscape both at baseline and after vedolizumab treatment. We observed highly dynamic changes within the innate and adaptive immune cell compartments during treatment. Using machine learning, we identified common features associated with vedolizumab treatment and effectively classified therapy response. Notably, a specific T-cell signature was linked to vedolizumab failure, independent of key clinical variables, such as treatment history or paraclinical parameters. Our findings provide novel insights into the immunologic mechanisms underlying therapy response and failure in patients with IBD treated with vedolizumab.

**WHAT YOU NEED TO KNOW**

BACKGROUND AND CONTEXT

Patients with inflammatory bowel disease (IBD) have benefitted from new biologic treatments, including the anti-integrin $\alpha4\beta7$ blocker vedolizumab. However, many patients do not achieve full remission and there are no reliable markers to predict responses.

NEW FINDINGS

The circulating immune cell landscape was thoroughly characterized in patients with IBD before and after treatment with vedolizumab, integrating multiparametric flow cytometry, mass cytometry, and serum proteomis (Olink) data into a predictive model. A predictive signature of Ki67$^+$ memory CD4$^+$ T cells (area under the curve, 0.97) was increased in nonresponders before treatment, exhibited an activated T-helper 1/T-helper 17 cell phenotype, and was not sufficiently targeted by vedolizumab.

LIMITATIONS

The findings were limited by the modest sample size and need multicenter validation. In addition, the findings were limited to circulating immune cells and not tissues and lack definitive proof of mechanism.

CLINICAL RESEARCH RELEVANCE

Ki67 expression on circulating CD4$^+$ memory T cells is a novel pretherapeutic nonresponse feature. This paves the way for a peripheral blood assay for precision therapy in patients with IBD.

BASIC RESEARCH RELEVANCE

This study provides new insights into immune cell characteristics after $\alpha4\beta7$ integrin blockade and associated with treatment failure. The findings are particularly relevant in the context of gut homing and recirculation of activated T cells.

*** Authors share co-first authorship.**

***Abbreviations used in this paper:*** **AUC, area under the curve; BCR, B-cell receptor; CD, Crohn's disease; CITEseq, cellular indexing of transcriptomes and epitopes by sequencing; CyTOF, cytometry by time of flight; FACS, fluorescence-activated cell sorting; IBD, inflammatory bowel disease; IL, interleukin; ITGB7, integrin subunit $\beta7$; MAdCAM-1, mucosal vascular addressin cell adhesion molecule 1; PBMC, peripheral blood mononuclear cell; scRNAseq, single-cell RNA sequencing; TCR, T-cell receptor; Th, T-helper; UC, ulcerative colitis; VCAM-1, vascular cell adhesion molecule–1.**

**Most current article**



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## Materials and Methods

### Study Design and Approval

A total of 47 patients (cohort 1; Supplementary Table 1) and 26 patients (cohort 2; Supplementary Table 7) receiving vedolizumab at the outpatient and inpatient clinic of the Department of Gastroenterology, Infectiology, and Rheumatology at Charité University Medicine Berlin were enrolled in either a prospective study (VEPREDEX#EA4/162/17) or a prospective biobank (IBDome-study; EA4/162/17), which were approved by the Charité University Medicine Berlin ethics committee. Donors in the first cohort were recruited between July 2018 and November 2021, and patients in the second cohort were included from December 2021 to July 2024. Eligible patients were aged 18–80 years with an established diagnosis of UC or CD. These patients were switched to vedolizumab due to persistently active disease, despite attempts at corticosteroid tapering, or due to a lack of response or intolerance to immunosuppressive medications (such as azathioprine) or tumor necrosis factor antagonists, even after appropriate induction and more than 4 weeks of treatment. Only 6 patients from both cohorts had received ustekinumab before vedolizumab, and none had been treated with JAK inhibitors before starting vedolizumab (Supplementary Table 16 and Technical Supplementary Figure 10). We also included 41 age- and sex-matched healthy donors (healthy controls) for comparison. All donors provided informed written consent for their participation in the study. In addition to biosampling, we collected relevant (para-) clinical data, including Harvey-Bradshaw Index/partial Mayo Score, C-reactive protein levels, and leukocyte and thrombocyte counts. Responders were classified as patients who achieved a minimum reduction of 3 points in the Harvey-Bradshaw Index and at least 2 points in the partial Mayo score after 30 weeks of vedolizumab treatment.

### Peripheral Blood Mononuclear Cell Thawing and Stimulation

PBMCs were thawed in a 37°C water bath, transferred to thawing medium, centrifuged, and resuspended for counting. Cells were used for flow cytometry, surface and transcription factor staining, and stimulated with phorbol myristate acetate/ionomycin for 4 hours. For details, see Supplementary Experimental Procedures.

### Flow Cytometry

PBMCs were stained according to standard protocols. For details, see Supplementary Experimental Procedures.

### Inhibition of Vascular Cell Adhesion Molecule–1 Binding in Memory CD4+ T Lymphocytes

The assay was done as described previously by Soler et al.[12] For details, see Supplementary Experimental Procedures.

### Mass Cytometry and Data Processing

Whole blood samples were fixed with PROT1 proteomic stabilizer and stored at −80°C. Upon thawing, samples were stained and acquired in batches of 15, including all time points from the same patient and matched healthy controls. For details, see Supplementary Experimental Procedures.

### Single-Cell Sequencing of Peripheral Blood Mononuclear Cells and Data Analysis

Following manufacturer's instructions, single-cell RNA sequencing (scRNAseq) libraries were generated using the Chromium Next GEM Single Cell 5' Reagent Kits, version 2, from 10x Genomics (Pleasanton, CA; CG000330 Rev D). Sequencing libraries for gene expression and T-cell receptor (TCR)/B-cell receptor (BCR) were processed together using Cell Ranger multi (version 5.0.0) and the GRCh38 genome annotation and analyzed using Seurat, version 4.0.11.[31]

### Proteomics Serum Assay

Proteomics analysis of patient serum samples was performed using the Olink Target 96 Inflammation panel platform (https://www.olink.com/products/inflammation/).

### Machine Learning

We applied machine learning techniques to identify markers across 4 data modalities for predicting vedolizumab efficacy in patients with IBD, using logistic regression and cross-validation. The top 10% most influential features were selected, with iterative model validation used to test predictive capacity. Performance was evaluated using area under the curve (AUC) and feature regularization methods (Lasso, ElasticNet) were applied for robustness. For details, see Supplementary Experimental Procedures.

### Code Availability

All original code necessary to replicate our analyses, including scRNAseq analyses and machine learning code, are deposited in a GitHub repository (https://github.com/VeroHo/vedo_paper).

### Data Availability

All source data relevant to understanding and reproducing the results presented in this article are provided in the Supplementary Material. Sequencing data (scRNAseq, CITEseq, and TCR sequencing) can be accessed at Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo) under accession number GSE261334.

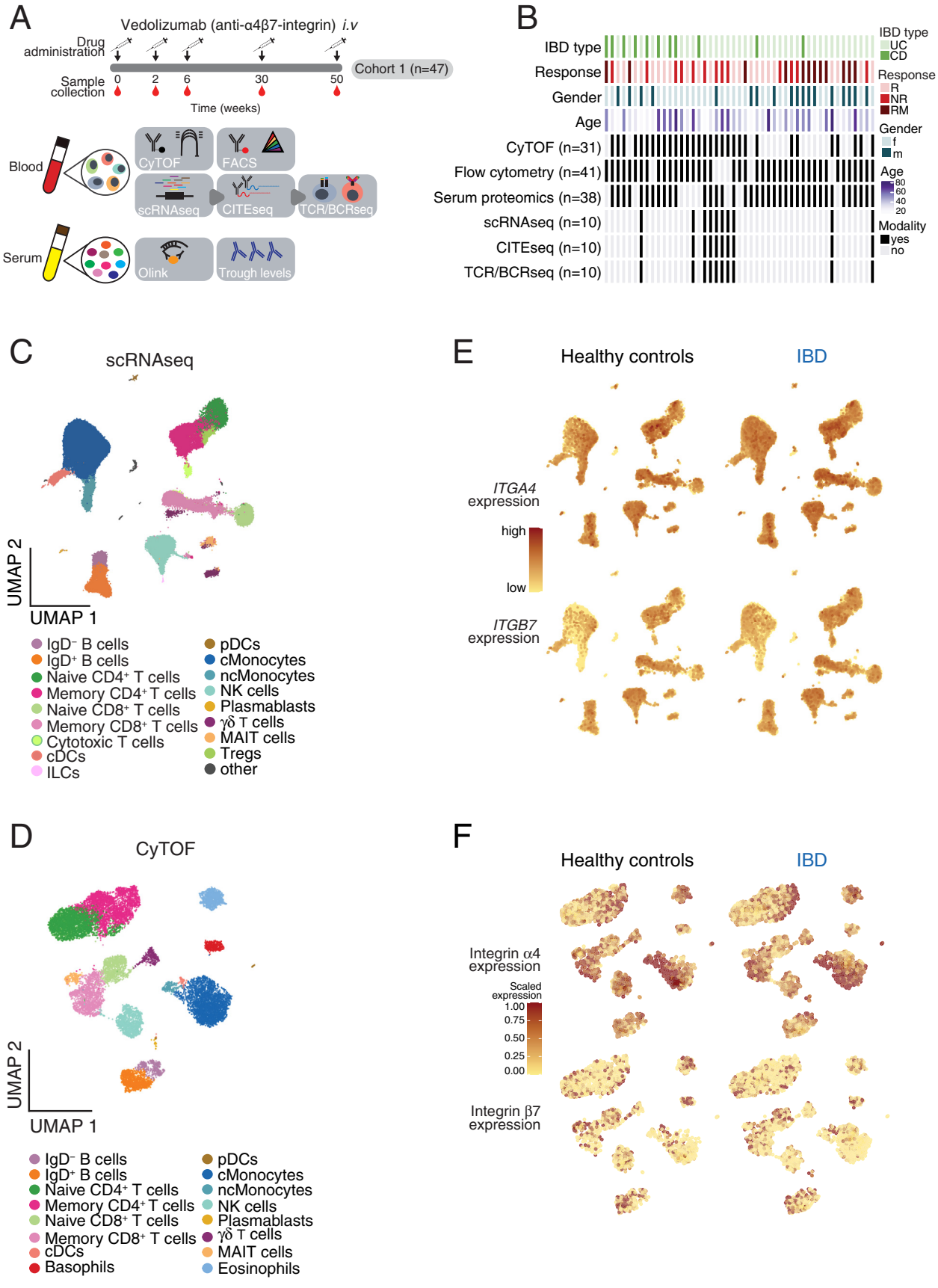
### Statistical Analysis

Statistical analyses and visualizations were created in R, using ggpubr (version 0.6.0), ggplot2 (version 3.4.3), limma (version 3.50.3), corrplot (version 0.92), and ComplexHeatmap (version 2.10.0) packages or with Prism software (GraphPad Software). *P* values were calculated using the Wilcoxon test. Paired analyses are indicated by connecting lines.

## Results

### Multimodal Profiling of Patients With Inflammatory Bowel Disease Receiving Anti-Integrin $\alpha4\beta7$ Therapy

We aimed to investigate the effect of vedolizumab treatment on circulating immune cells at baseline and during treatment. Therefore, we prospectively sampled blood from a cohort of patients with IBD receiving vedolizumab at


A
Vedolizumab (anti-α4β7-integrin) i.v
Drug administration
Sample collection
0
2
6
30
50
Cohort 1 (n=47)
Time (weeks)
Blood
CyTOF
FACS
scRNAseq
CITEseq
TCR/BCRseq
Serum
Olink
Trough levels
B
IBD type
Response
Gender
Age
CyTOF (n=31)
Flow cytometry (n=41)
Serum proteomics (n=38)
scRNAseq (n=10)
CITEseq (n=10)
TCR/BCRseq (n=10)
IBD type
UC
CD
Response
R
NR
RM
Gender
f
m
Age
80
60
40
20
Modality
yes
no
C
scRNAseq
UMAP 2
UMAP 1
IgD⁻ B cells
IgD⁺ B cells
Naive CD4⁺ T cells
Memory CD4⁺ T cells
Naive CD8⁺ T cells
Memory CD8⁺ T cells
Cytotoxic T cells
cDCs
ILCs
pDCs
cMonocytes
ncMonocytes
NK cells
Plasmablasts
γδ T cells
MAIT cells
Tregs
other
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CyTOF
UMAP 2
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IgD⁻ B cells
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Plasmablasts
γδ T cells
MAIT cells
Eosinophils
E
Healthy controls
IBD
ITGA4 expression
high
low
ITGB7 expression
F
Healthy controls
IBD
Integrin α4 expression
Scaled expression
1.00
0.75
0.50
0.25
0.00
Integrin β7 expression

baseline and during treatment (IBD, n = 47; Figure 1*A*, Supplementary Table 1) together with age- and sex-matched healthy controls (n = 41; Supplementary Figure 1*A*, Supplementary Table 1). We recorded disease activity scores, prior treatments, and paraclinical parameters (Supplementary Figure 1*B*–*E*, Supplementary Table 16). Responders were classified as patients who achieved a minimum reduction of 3 points in the Harvey-Bradshaw Index (for CD) and at least 2 points in the partial Mayo score (for UC) after 30 weeks of vedolizumab treatment and continued vedolizumab treatment. Stool calprotectin levels at 18 weeks effectively distinguished responders from nonresponders (Supplementary Figure 1*D*).

Mass cytometry was used to profile whole blood, identifying and characterizing immune cell subsets of major innate and adaptive populations with 36 protein targets (Supplementary Tables 10 and 11). Serum samples underwent proteomic analysis using the Olink proximity extension assay (Target 96 Inflammation panel, Supplementary Table 14) to detect changes in inflammatory markers before and 6 weeks after treatment induction (Figure 1*A* and *B*). In a selected sub-cohort, PBMCs were analyzed using single-cell sequencing and immune repertoire profiling to evaluate gene and protein expression (CITEseq) and to monitor changes in TCR and BCR repertoires upon treatment (Supplementary Tables 12 and 13). In addition, multiparametric flow cytometry (fluorescence-activated cell sorting [FACS]) was used to examine the expression of chemokine receptors, surface molecules, integrins, cytokines, and transcription factors in circulating T cells (Figure 1*B*, Supplementary Tables 8 and 9).

## *Integrin α4β7 Expression and Distribution on Circulating Immune Cells at Steady-State and After Treatment*

We first obtained a comprehensive overview of the circulating immune cell landscape in healthy controls and patients with IBD. CITEseq analysis and mass cytometry identified approximately 16–18 different cell clusters (Figure 1*C* and *D*, Supplementary Figures 2*A* and *B* and 3*A* and *B*). Both datasets revealed that patients with IBD have an altered immune cell composition compared with healthy controls (Supplementary Figures 2*C* and 3*C*). Next, we performed a comprehensive single-cell analysis of α4β7 integrin expression in both healthy and diseased conditions (Figures 1*E* and *F* and 2*A* and *B*). The scRNAseq analysis revealed *ITGA4* (integrin α4) and *ITGB7* (integrin β7) expression across all lineages (Figure 1*E* and 2*A*). *ITGA4* showed high expression across all major cell types (Figures 1*E* and 2*A*), although there was differential *ITGB7* expression on specific immune cells (Figures 1*E* and 2*A*). *ITGA4* and *ITGB7* expression were similar between healthy controls and patients with IBD (Figure 2*A* and Supplementary Figure 4*A*).

We next explored the protein expression of integrin α4 and β7 on circulating immune cells to identify cell lineages potentially targeted by vedolizumab. In our CITEseq dataset, integrin β7 protein expression (ADT, antibody-derived tag) showed a similar distribution to *ITGB7* RNA expression (Figure 2*B*). Gating on α4β7 co-expressing cells in the mass cytometry dataset and performing cell clustering within this subset (Figure 2*C* and Supplementary Figure 4*B*), we found that α4 and β7 integrins were co-expressed not only in T cells and monocytes, but also in other cell lineages, with CD4/CD8$^+$ T cells and eosinophils being the most abundant (Figure 2*C*). The frequency of α4β7$^+$ cells was significantly reduced in patients with IBD before treatment induction across several cell types (Supplementary Figure 4*C* and *D*). Surprisingly, protein expression of integrin β7, but not integrin α4, was up-regulated after vedolizumab treatment in all cell subsets (Figure 2*B* and *D*), with significant changes in memory CD4$^+$ and CD8$^+$ T cells, eosinophils, classical monocytes, memory B cells, basophils, conventional dendritic cells, and natural killer cells (Figure 2*E*).

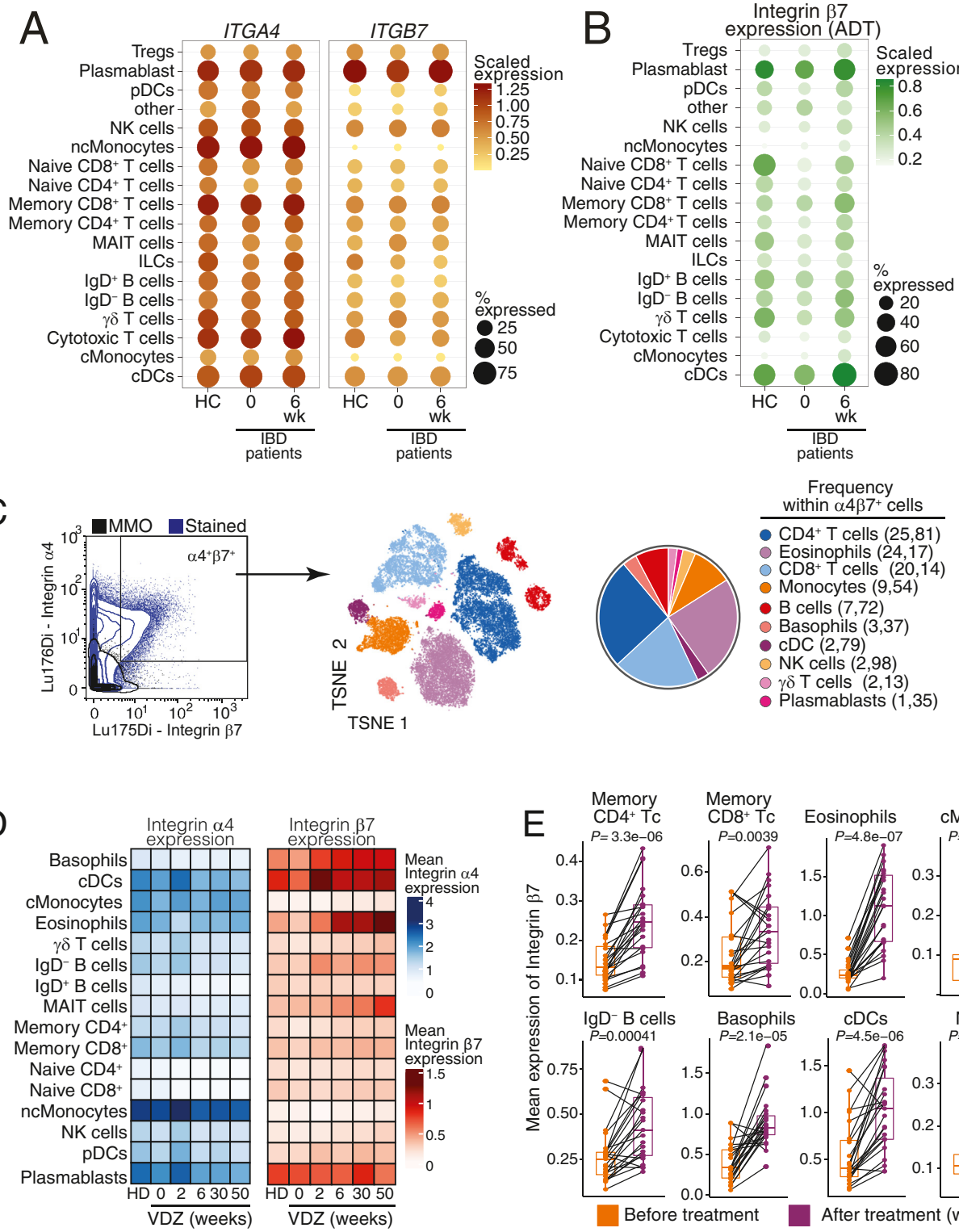
Taken together, our data demonstrated that integrin α4β7 is expressed on various circulating cell lineages and that vedolizumab modulates expression of integrin β7.

## *Vedolizumab Enhances Clonal Diversity in Circulating Memory T Cells*

Targeting memory T cells expressing integrin α4β7 is a key mechanism by which vedolizumab modulates intestinal inflammation.[13] Animal studies suggest that anti-integrin α4β7 treatment releases gut-resident immune cells into circulation.[5,11,14] To test this, we assessed the diversity of circulating T and B cells before and after treatment using single-cell TCR and BCR repertoire analysis (TCR/BCR sequencing) combined with CITEseq (Figure 3*A* and *B*, Supplementary Figure 5*A* and *F*). We found a significant increase in clonal diversity, specifically in memory CD4$^+$ T cells, but not CD8$^+$ T cells and B-cell subsets after treatment

**Figure 1.** Multimodal characterization of patients with IBD receiving anti-integrin α4β7. (*A*) Study design. (*B*) The study cohort and analyses applied to each patient sample (UC, CD; response: responder [R], nonresponder [NR], remission maintenance [RM]; gender, age). (*C*) Uniform Manifold Approximation and Projection (UMAP) plots of CITEseq profiled peripheral blood cells with color-coded representation of cell types and patients (n = 191,578 cells derived from a total of 25 samples: healthy controls [HC], n = 5; IBD, n = 10 matched before and after treatment). Sorted CD45$^+$ cells from the peripheral blood of 5 healthy controls and 10 patients with UC before and 6 weeks after vedolizumab (VDZ) treatment initiation were subjected to CITEseq analysis. cDC, conventional dendritic cell; ILC, innate lymphoid cell; MAIT, mucosal-associated invariant T cell; NK, natural killer; pDC, plasmacytoid dendritic cell; Treg, regulatory T cell. (*D*) UMAP plots of peripheral blood cells extracted from the CyTOF dataset, with color representation based on cell type and patient. Results derived from FlowSOM/ConsensusPlus clustering analysis performed on 13,311,287 cells from 154 samples. This dataset includes both HCs and patients with IBD both before and during treatment. (*E*) Expression of *ITGA4* and *ITGB7* in the different cell subsets derived from scRNAseq data. (*F*) Scaled expression of integrin α4 and β7 in the CyTOF dataset from HCs and patients with IBD before therapy (IBD).

(Figure 3*C*, Supplementary Figure 5*B* and *G*). After treatment, there was an increase in TCR diversity, specifically in central memory CD4$^+$ T cells, but not in effector memory CD4$^+$ T cells or in the corresponding CD8$^+$ T-cell populations (Figure 3*D* and Supplementary Figure 5*B*). Using the scRNAseq dataset, we investigated whether anti-integrin $\alpha4\beta7$ treatment specifically affects TCR diversity in gut-homing T-cell subsets. We found a significant increase in TCR diversity in CD4$^+$ T cells expressing *ITGA4* and *ITGB7*, but not in those expressing *ITGB1*, *GPR15*, or *ITGAE*



(Figure 3*E* and *F* and Supplementary Figure 5*A*). However, treatment did not increase TCR diversity among gut-homing CD8$^+$ T cells (Supplementary Figure 5*C* and *D*). There were no specific alterations in TCR and BCR diversity based on therapy response (Supplementary Figure 6*A* and *B*). Consistent with the increase in TCR diversity of CD4$^+$ T cells, memory CD4$^+$ T cells (but not CD8$^+$ or B cells) also significantly increased after treatment (Figure 3*G–I*, Supplementary Figure 5*E*, *H*, and *I*).

These findings provide evidence that vedolizumab significantly increases clonal diversity within circulating memory T cells, with a particular impact on central memory and $\alpha4\beta7^+$ memory CD4$^+$ T cells.

## *Vedolizumab Modulates the Abundance of Circulating $\alpha4\beta7$-Integrin$^+$ Immune Cells and Proinflammatory Serum Proteins*

We next integrated our multimodal datasets into a reductionist machine learning model, focusing on cytometry by time of flight (CyTOF), FACS, and Olink data to gain a comprehensive understanding of vedolizumab's immunologic effects in the circulation of patients with IBD. Due to observed changes in their TCR diversity, we focused our flow cytometry analysis on memory CD4$^+$ T cells (Figure 4*A*, Supplementary Table 2, Supplementary Figures 7 and 8).

Using a logistic regression classifier, we found that vedolizumab treatment significantly increased the abundance of integrin $\alpha4\beta7$ on circulating immune cells (CyTOF-$\alpha4\beta7$; AUC, 0.97; Figure 4*B*). The abundance of $\alpha4\beta7^+$ cells in the bloodstream increased over time with vedolizumab treatment (Figure 4*C*), especially in populations with the highest integrin $\alpha4\beta7$ expression (Figures 4*D* and *E*). The second major effect of vedolizumab was observed in inflammatory serum protein levels at week 6 after treatment initiation (Olink; AUC, 0.94; Figure 4*B*). Unexpectedly, several serum proteins, including eotaxin (CCL11), delta/notch-like epidermal growth factor-related receptor, chemokine (C-X3-C motif) ligand (CX3CL) 1, tumor necrosis factor–$\beta$ (or LT-$\beta$), and chemokine (C-C motif) ligand (CCL) 28, showed significant increases after vedolizumab treatment (Figures 4*F* and *G*, Supplementary Figure 9*A–C*). To explore the mechanism underlying this phenomenon, we analyzed gene expression corresponding to the inflammatory serum proteins in the intestinal mucosa of patients with IBD treated with vedolizumab in RNAseq data from the GEMINI trial (GSE73661).[10] In the intestinal mucosa, the genes corresponding to the Olink proteins were not upregulated after vedolizumab treatment, suggesting that the increased protein serum levels cannot be attributed to enhanced expression of these genes in inflamed tissue (Supplementary Figure 10*A–C*). However, we found a positive correlation between the top serum markers and the abundance of the most altered $\alpha4\beta7$-expressing populations identified by mass cytometry, suggesting that immune cell entrapment after anti-integrin treatment may explain the increased serum inflammatory markers (Figure 4*H*).

In conclusion, vedolizumab strongly alters the abundance of circulating immune cells, particularly those expressing high levels of $\alpha4\beta7$ integrin, and this is associated with increased inflammatory serum markers.

## *Integration of Multimodal Data Identifies Determinants of Nonresponse to Anti-Integrin Treatment*

We applied both linear and nonlinear classification approaches to the multiparametric data (Figure 5*A*, Supplementary Figure 11*A* and *B*, and Supplementary Table 15) to identify features associated with vedolizumab nonresponse. After evaluating performance and model simplicity, we selected logistic regression as the final method for classifying treatment outcomes (Supplementary Figure 11*A–C*, Supplementary Table 3).

Surprisingly, when we analyzed all measured parameters (features) from individual modalities (eg, FACS, Olink, CyTOF, CyTOF-$\alpha4\beta7$), we were unable to successfully classify therapy outcomes (Figure 5*B* and Supplementary Figure 11*D*, *upper left*, Supplementary Table 3). In addition, combining all modalities did not improve classification performance (Figure 5*B* and Supplementary Figure 11*D*, *lower left*). However, selecting the top 10% of features from each modality significantly improved classification accuracy (Figure 5*B* and Supplementary Figure 11*E*). We achieved an AUC of 0.89 for Olink and 0.91 for FACS individually (Figure 5*B* and Supplementary Figure 11*D*, *upper right*). Combining the top 10% of features from both modalities further improved performance, reaching an AUC of 0.98 (Figure 5*B* and Supplementary Figure 11*D*, *lower right*).

Testing the 4 best-performing models using an iterative approach resulted in the validation of the models containing

**Figure 2.** Integrin $\alpha4\beta7$ expression and distribution in circulating immune cells at steady-state and modulation upon anti-integrin $\alpha4\beta7$ treatment. (*A*) Dot plot of *ITGA4* and *ITGB7* expression in scRNAseq data from different cell types and different conditions before treatment (0) and 6 weeks (6 wk) after treatment. *Color* indicates expression level, *dot size* indicates the percentage of positive cells. cDC, conventional dendritic cell; ILC, innate lymphoid cell; MAIT, mucosal-associated invariant T cell; NK, natural killer; pDC, plasmacytoid dendritic cell; Treg, regulatory T cell. (*B*) As in (*A*), derived from integrin $\beta7$ protein expression (antibody-derived tag [ADT]) by CITEseq. (*C*) Representative dot plot of integrin $\alpha4$ (CD49d) and $\beta7$ staining in mass cytometry on CD45$^+$ cells showing stained cells (*blue*) and metals-minus-one (MMO) control. T-distributed stochastic neighbor embedding (TSNE) with results of FlowSOM clustering of $\alpha4\beta7^+$ cells from the mass cytometry dataset and a pie chart representing the percentage of cluster frequency within the total $\alpha4\beta7^+$ cells derived from healthy controls (HCs) and patients with IBD. (*D*) Mean integrin $\alpha4$ and $\beta7$ expression within mass cytometry clusters, as determined by the FlowSOM algorithm in healthy donors (HDs) and patients with IBD before treatment and at the indicated time points after treatment initiation (HDs, n = 27; before treatment, n = 31; week 2, n = 9; week 6, n = 25; week 30, n = 15; week 50, n = 9). (*E*) Comparison of mean integrin $\beta7$ expression in indicated mass cytometry cluster before and 6 weeks after treatment. *P* values were calculated using the paired Wilcoxon test.

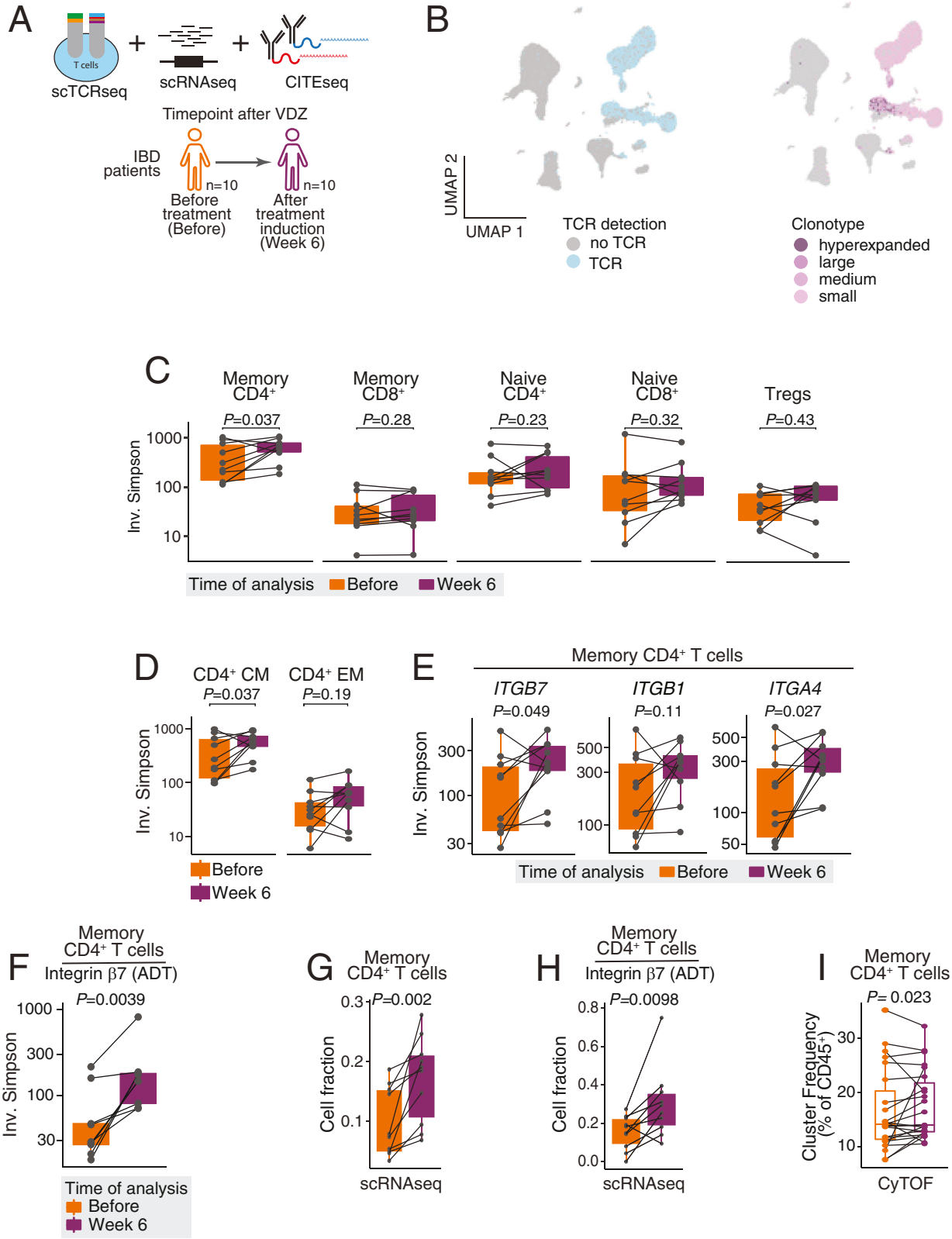
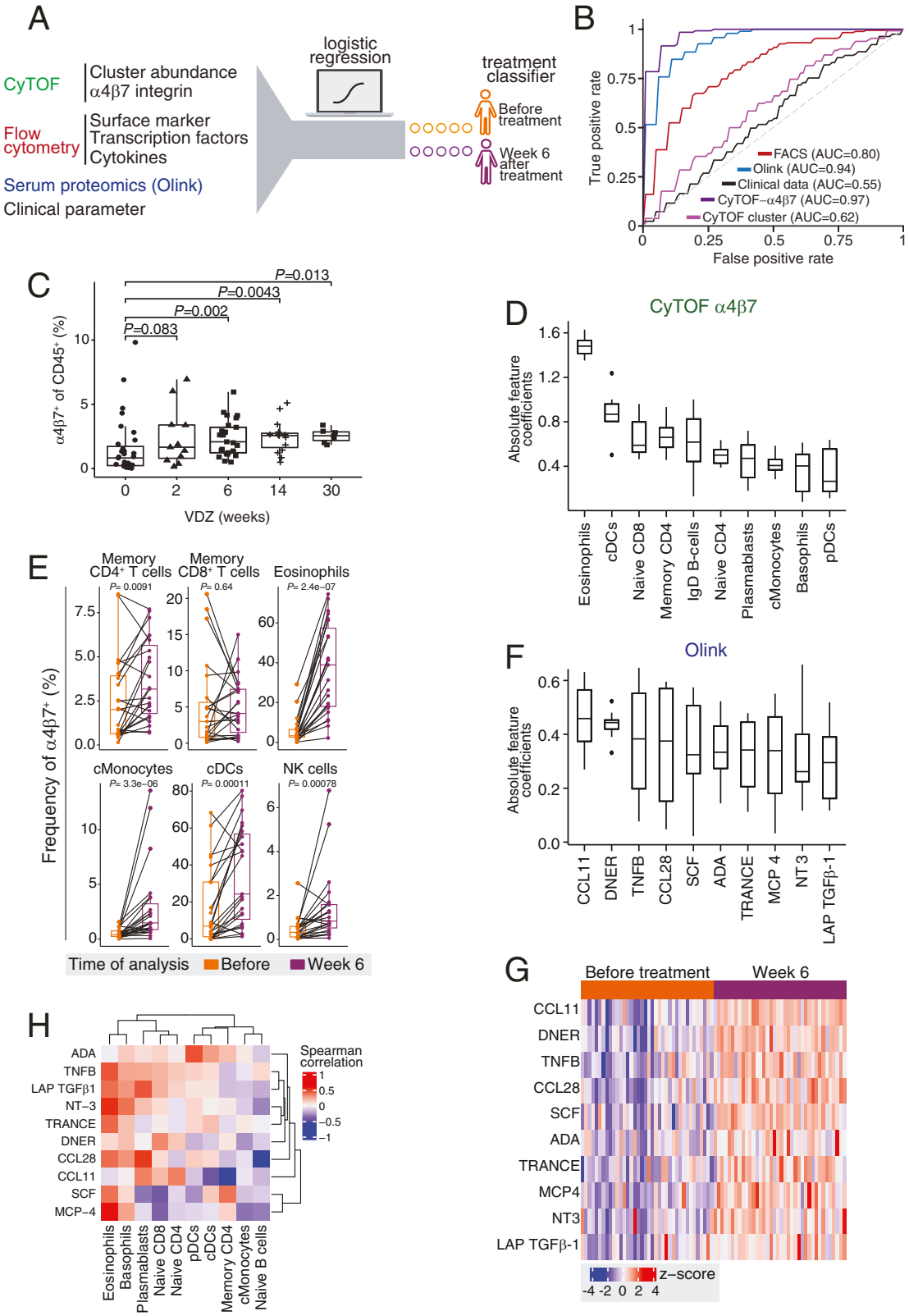



**Figure 3.** Vedolizumab enhances clonal diversity among circulating CD4$^+$ memory T cells. (*A*) Schematic of the experimental approach. (*B*) Uniform Manifold Approximation and Projection (UMAP) plot indicating cells with detected TCR sequence (*left*) and frequency of the associated clonotype (*right*; small: $< 10^{-3}$, medium: $<10^{-2}$, large: $<10^{-1}$, hyperexpanded: $>10^{-1}$). (*C*) Clonal diversity quantified by the inverse Simpson index in various T-cell subsets before treatment and 6 weeks after treatment. Treg, regulatory T cell. (*D*) Clonal diversity in central and effector memory CD4$^+$ T-cell subpopulations. (*E*) Clonal diversity in memory CD4$^+$ T-cell subpopulations defined by expression of the indicated marker genes. (*F*) Clonal diversity in memory CD4$^+$ T cells with positive integrin $\beta7$ expression (antibody-derived tag [ADT]) determined by CITEseq. (*G*) Abundance of memory CD4$^+$ T cells among all sequenced cells per sample. (*H*) Abundance of memory CD4$^+$ T cells with positive integrin $\beta7$ expression determined by CITEseq. (*I*) Cluster frequency in the mass cytometry dataset. (*C–I*) Wilcoxon test.

(1) Olink/FACS, (2) Olink/FACS/CyTOF, and (3) FACS/CyTOF/clinical parameters, although FACS/CyTOF-α4β7 did not reach the required test accuracy >0.5 (Supplementary Figure 12*A*). For each of the 3 validated models, we selected the top 5 features with highest absolute feature coefficients (Figure 5*C* and Supplementary Figure 12*B*), which also emerged from a similar analysis using automatic feature selection methods (Supplementary Figure 12*C*,



Supplementary Table 4), and used the resulting refined models for classification of treatment response (Figure 5*D*). Parameters within memory CD4$^+$ T cells, including chemokine receptors, cytokines, serum inflammatory markers, abundance of natural killer cells in the circulation measured by mass cytometry, and especially the proliferation marker Ki67, had a high influence on predictive capacity. An AUC of 0.99 was achieved by selecting a panel containing the top 3 features in the FACS/Olink/CyTOF model (Figure 5*D*), while still maintaining test set accuracies of 0.75. Here, classification performance remained compelling even when tested on a reduced patient set (Supplementary Figure 12*D*, Supplementary Table 5). Remarkably, evaluating the predictive capacity of Ki67$^+$ memory CD4$^+$ T cells alone in the FACS/Olink/CyTOF model yielded an AUC of 0.93, thus scoring higher than the majority of top-3 and top-5 feature combinations.

To test the reproducibility of these findings, we next recruited a validation cohort of patients with IBD undergoing vedolizumab treatment (n = 26) and analyzed PBMCs using an optimized and simplified flow cytometry panel. In that cohort, the combination of our top 3 FACS markers (Ki67, CXCR3, and IL4) achieved an AUC of 0.99 (Figure 5*E*).

Thus, our 3-feature FACS panel on Ki67$^+$ memory CD4$^+$ T cells allowed reliable prediction of treatment response, and we identified the abundance of proliferating CD4$^+$ memory T cells in circulation as a key classifier of vedolizumab treatment outcome.

## *Transcriptional Characteristics of Circulating Ki67$^+$ Effector CD4$^+$ T Cells*

To further investigate the observed increase in T-cell proliferation in vedolizumab nonresponders, we analyzed different T-cell populations for Ki67 expression. We observed significant enrichment of Ki67$^+$ cells within total CD3$^+$ T cells in the blood of nonresponders (Supplementary Figure 13*A*). This increased Ki67$^+$ fraction was specific to memory CD4$^+$ T cells (but not regulatory or memory CD8$^+$ T cells) in nonresponders compared with responders in both patients with UC and patients with CD (Supplementary Figure 13*A*–*C*). This finding was also confirmed in the validation cohort (Supplementary Figure 13*D*). Interestingly, Ki67$^+$ levels in memory CD4$^+$ T cells were significantly elevated in patients with IBD before therapy initiation and remained unaffected by vedolizumab (Supplementary Figure 13*E* and *F*).

To further characterize Ki67$^+$ memory CD4$^+$ T cells, we conducted additional single-cell sequencing analysis on FACS sort-purified memory CD4$^+$ T cells from the same donors used initially in our study, which was integrated with the pre-existing PBMC scCITEseq data (Figure 6*A* and Figure 1*B*). Hierarchical clustering resulted in 12 distinct clusters within the sequenced memory CD4$^+$ T cells (Figure 6*B*, Supplementary Figure 14*A*–*C*), with predominant enrichment of Ki67$^+$ memory CD4$^+$ T cells in cluster 1 (Figure 6*C* and *D*), which, consistent with our FACS data, was significantly increased in nonresponders before treatment (Figure 6*E*).

We compared the genes expressed in cluster 1 with all other clusters (Figure 6*F*, Supplementary Table 6), focusing on classical activation and homing markers, cytokine receptors, cytokines, and classical T cell–related transcription factors (Figure 6*G*). Our analysis revealed signs of activation and proliferation in cluster 1 (up-regulated *HLADRA, CD40LG,* and *MKI67*, down-regulated *IL7R* and *SELL*) (Figure 6*G*, activation markers, and cytokine receptors). Furthermore, cells in cluster 1 showed high *ITGA4, ITGB1*, and *ITGB7* levels, and *CXCR3* and CCR6 (Figure 6*G*, homing receptors) expressed *IL12BR2*, *IL18R1,* and showed down-regulation of *IFNGR2* (Figure 6*G*, cytokine receptors). Several transcription factors, including *TBX21, RORC, MAF*, and *TOX,* were up-regulated (Figure 6*G*, transcription factors). By integrating protein data, we confirmed that cluster 1 expressed increased protein levels of HLA-DR, while displaying low expression of CD25, CD45RA, and CD127 (Figure 6*H*). Our findings indicate that Ki67$^+$ memory CD4$^+$ T cells constitute an activated subpopulation distinguished by T-helper (Th) 1 and Th17 attributes, elevated integrin expression, heightened gut-homing potential, and increased activation markers.

## *Proliferating Effector Memory CD4 T Cells Exhibit Expression of Alternative Homing Receptors and T-helper 1/T-helper 17 Cell Characteristics*

We used multiparametric flow cytometry to explore the functional characteristics of the predictive Ki67$^+$ effector memory CD4$^+$ T-cell signature in IBD, including markers identified by single-cell sequencing. There was a positive

**Figure 4.** Vedolizumab (VDZ) modulates integrin $\alpha4\beta7$ expression on circulating immune cells, cell type abundance, and proinflammatory serum proteins. (*A*) Machine learning approach used to identify the effects of VDZ treatment on circulating immune cells. (*B*) Capacity of clinical parameters, Olink, mass cytometry, and flow cytometry data to display VDZ treatment signatures shown as receiver operating characteristic curves (ROC) with corresponding AUC values. (*C*) Percentage of $\alpha4\beta7^+$ cells from total CD45$^+$ cells in the mass cytometry dataset at different time points throughout treatment (patients with IBD before treatment, n = 31; week 2, n = 9; week 6, n = 25; week 30, n = 15; week 50, n = 9). Mann-Whitney test. (*D*) Overview of the top 10 VDZ efficacy features from the CyTOF-$\alpha4\beta7$ dataset with the largest feature coefficients. These populations have the highest feature coefficients, and the absolute values of these coefficients are shown. The complete list can be found in Supplementary Table 3. cDC, conventional dendritic cell; pDC, plasmacytoid dendritic cell. (*E*) Percentage of $\alpha4\beta7^+$ cells from total CD45$^+$ per indicated cluster in the mass cytometry dataset from week 0 vs week 6 (patients with IBD before treatment, n = 31; after treatment, n = 25). Paired Wilcoxon test. NK, natural killer. (*F*) Overview of the 10 VDZ efficacy features from Olink data with the largest feature coefficients. DNER, delta/notch-like epidermal growth factor-related receptor. (*G*) Olink quantification of the indicated analytes before treatment (n = 39) and 6 weeks after VDZ treatment (n = 39). (*H*) Spearman correlation between the serum proteomics' most significantly altered cytokines and the abundance of cell populations measured by CyTOF after treatment.

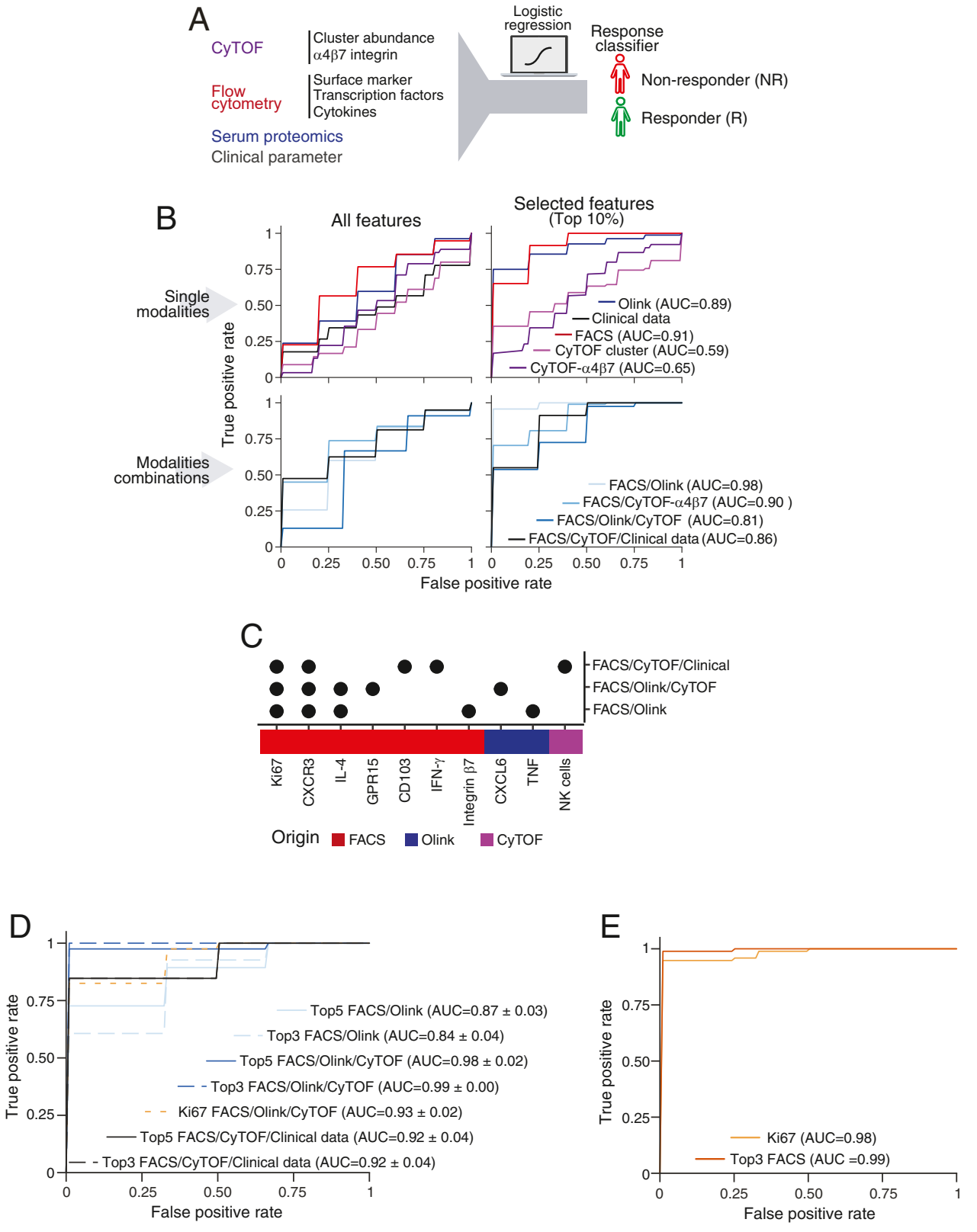
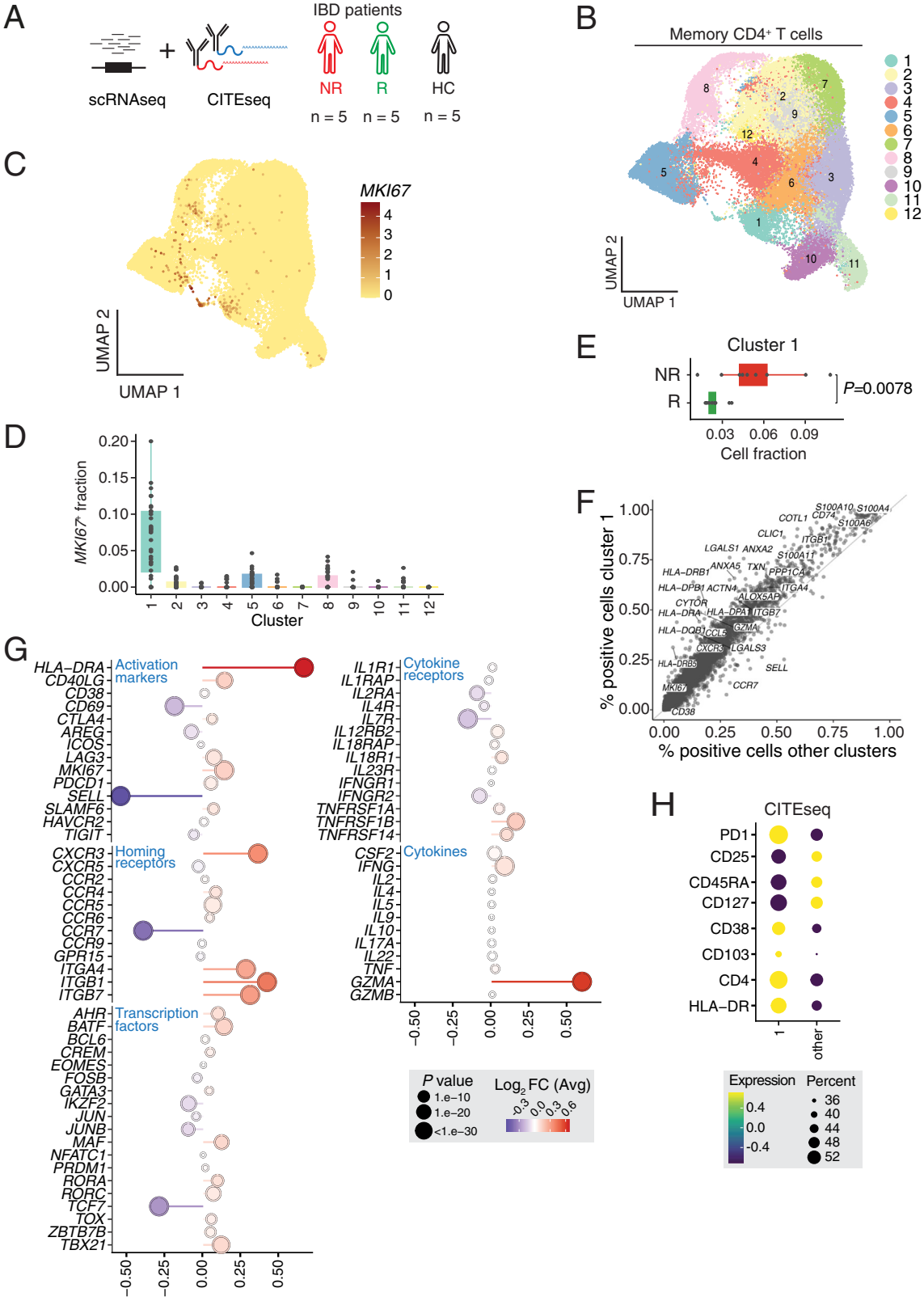



**Figure 5.** Machine learning–based classification identifies vedolizumab-induced immune changes and classifies therapy response. (*A*) Machine-learning approach used to identify the effects of vedolizumab treatment on circulating immune cells and to classify treatment response. (*B*) Predictive capacity of individual data sets (*top*) and data combinations showing the highest AUC values (*bottom*) to classify vedolizumab response. Classifications are performed using all features (*left*) or the top 10% most predictive features from the ranked logistic regression coefficients (*right*). (*C*) Occurrence of the 5 most predictive features across the validated best-performing models, see also Supplementary Figure 12*B*. IFN, interferon; NK, natural killer; TNF, tumor necrosis factor. (*D*) Predictive capacity of selected marker combinations from the validated top-performing models (cf Supplementary Figure 12*A*). (*E*) Biomarker validation in an independent cohort of patients with IBD treated with vedolizumab (n = 26). As indicated, the predictive capacity is shown using Ki67 or the top 3 most frequent FACS markers (ie, Ki67, CXCR3, and IL-4).

correlation between Ki67 and HLA-DR, CD38, and PD-1 expression (Supplementary Figure 15*A*). Ki67$^{+}$ memory CD4$^{+}$ T cells showed increased expression of HLA-DR, CD38, and PD-1 and decreased expression of CD127 and CCR7 compared with Ki67$^{-}$ cells (Figure 7*A–C*; Supplementary Figure 15*A–C*). These cells also expressed high levels of





integrin $\alpha 4$ and $\beta 1$, with nearly 80% expressing integrin $\alpha 4\beta 1$ and 20% expressing integrin $\alpha 4\beta 7$ (Figure 7*A*–*C*). Indeed, vedolizumab did not inhibit interactions between integrin $\alpha 4\beta 1$ and its ligand vascular cell adhesion molecule (VCAM) (Supplementary Figure 15*E*–*G*). In addition, Ki67$^+$ memory CD4$^+$ T cells expressed high levels of GPR15, CXCR3, and CCR6, which are critical for migration to inflamed sites (Supplementary Figure 15*B* and *C*), consistent with the high expression of corresponding chemokine ligands and adhesion molecules in inflamed intestinal tissue (Supplementary Figure 16).

Expression of Th1/Th17 transcription factors T-bet, Eomes, RORγt, and the cytokine IL-17A were also increased in Ki67$^+$ memory CD4$^+$ T cells (Figure 7*D* and *E*; Supplementary Figure 15*D*). In vedolizumab nonresponders, there was an increase in T-bet$^+$RORγt$^+$ cells and a reduction in FoxP3 expression within Ki67$^+$ memory CD4$^+$ T cells (Figure 7*F*). These findings collectively demonstrate that Ki67$^+$ memory T cells are significantly enriched in the blood of patients who fail to respond to anti-integrin therapy, express $\alpha 4\beta 1$ integrin, and exhibit an activated Th1/Th17 phenotype.

## Discussion

This study investigated the impact of anti-integrin $\alpha 4\beta 7$ treatment on the circulating immune cell landscape in IBD and identified factors associated with nonresponse. First, $\alpha 4\beta 7$ integrin was expressed across various immune cell types, including both innate and adaptive circulating immune cells, leading to significant alterations in their composition and expression of $\alpha 4\beta 7$ integrin after therapy initiation. Second, the TCR repertoire of CD4$^+$ T cells, specifically effector and $\alpha 4\beta 7^+$ memory CD4$^+$ T cells, changed after treatment. Third, integrating acquired multimodal parameters into a composite model effectively classified therapy response to vedolizumab. Fourth, the predictive activated cycling effector CD4 T-cell subset expressed molecules associated with pathogenic Th1/Th17 responses, including the transcription factors T-bet and RORγt and homing markers, including CXCR3, CCR6, and integrin $\alpha 4\beta 1$. Finally, we confirmed that the abundance of circulating proliferating effector CD4$^+$ T cells is a novel classifier of vedolizumab failure in patients with IBD.

Vedolizumab is thought to reduce intestinal inflammation by modulating T cell migration and entry to the gut. Notably, gut-resident CD4$^+$ T cells can migrate from gut tissue into the circulation.[32] Vedolizumab treatment may disrupt their re-entry into the gut, possibly leading to retention of circulating gut-resident CD4$^+$ T cells in the peripheral blood. This phenomenon could account for the observed enhancement in clonal diversity among circulating CD4$^+$ T cells. However, its effects on the abundance and activation of intestinal T cells and the colonic TCR repertoire have been described as minor.[33]

Our findings reveal broad $\alpha 4\beta 7$ integrin expression on a variety of immune cell types, but $\alpha 4\beta 7$ integrin levels changed most significantly in circulating myeloid populations after treatment. Transcriptional analysis of whole colonic biopsies acquired before and after vedolizumab treatment previously suggested that vedolizumab primarily affects innate rather than adaptive immunity.[33] Indeed, inflammation-adapted emergency hematopoiesis, increased bone marrow output, and skewing toward enhanced granulocyte-monocyte progenitors have been found to occur and support intestinal inflammation, and these granulocytic populations express integrin $\alpha 4\beta 7$ and migrate to mucosal tissues.[34,35] Consistent with these data, we observed an increased abundance of circulating eosinophils, monocytes, and dendritic cells after vedolizumab treatment, irrespective of therapy response. These findings suggest that by targeting $\alpha 4\beta 7$ integrin, vedolizumab potentially reduces infiltration of newly generated myeloid cells into gut tissue.

Because the influence of vedolizumab treatment on the systemic inflammatory environment in patients remains unclear, we measured proinflammatory serum proteins before therapy initiation and 6 weeks later. We observed significant alterations in serum proinflammatory markers in patients with IBD compared with healthy controls. Of particular interest, we noted an increase in several serum markers after 6 weeks of vedolizumab, consistent with preclinical observations in rhesus macaques.[15] Interestingly, we found a strong correlation between the top-enriched serum analytes and the abundance of various cellular populations also enriched in peripheral blood after vedolizumab treatment. This suggests that the sequestration of inflammatory cells in the circulation may contribute to proinflammatory cytokine and chemokine release.

Ki67$^+$ memory CD4$^+$ T cells were enriched in patients with IBD compared with healthy controls, and these cells were significantly increased in nonresponders to vedolizumab. Furthermore, these cells co-expressed activation markers CD38 and HLA-DR, which are increased in the blood and colonic mucosa of patients with IBD.[36] Ki67$^+$ memory CD4$^+$ T cells showed reduced CD127 and CCR7 expression, while HLA-DR and CD38 expression was increased, confirming their activation and effector status. Indeed,



**Figure 6.** Enrichment of proliferating Ki67$^+$ effector CD4$^+$ T cells in vedolizumab-refractory patients. (*A*) Schematic depiction of experimental approach. (*B*) Uniform Manifold Approximation and Projection (UMAP) plot of CD4$^+$ memory cells using combined PBMC and CD4$^+$-sorted scRNAseq data from 5 HCs and 10 patients with IBD. (*C*) Normalized expression of *MKI67* in CD4$^+$ memory cells. (*D*) Fraction of *MKI67*$^+$ cells within different clusters. (*E*) Fraction of *MKI67*$^+$ cells in cluster 1 for responders (R) and nonresponders (NR), respectively. Mann-Whitney test. (*F*) Scatter plot of marker genes for cluster 1 showing percentage of cells with detectable expression in cluster 1 compared with all other clusters. (*G*) Lollipop graph illustrating fold expression of selected genes in cluster 1 compared with other clusters. The *color scale* represents the mean $\log_2$-fold expression, and the *dot size* represents the *P* value. Genes are categorized according to different functions, including activation markers, cytokine receptors, transcription factors, cytokines, and homing receptors. (*H*) Dot plot displaying scaled CITEseq expression of selected markers in cluster 1 in comparison with other clusters.

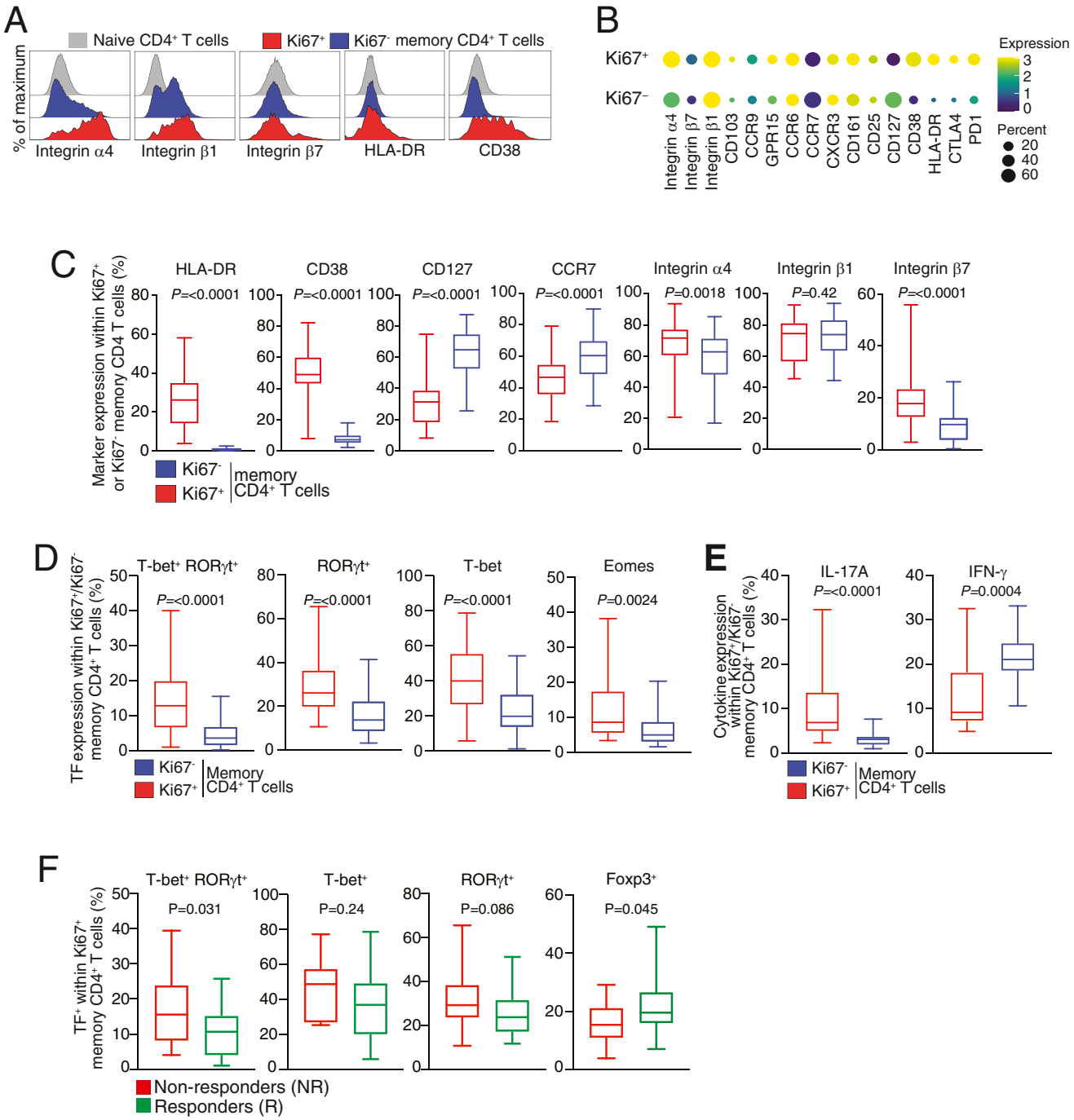



**Figure 7.** Distinctive features of proliferating effector CD4$^+$ T cells in vedolizumab nonresponsive patients. (*A*) Histograms of FACS surface marker expression in a representative patient with IBD. Shown is the expression of the indicated markers on naïve, Ki67$^+$, and Ki67$^-$ memory CD4$^+$ T cells. (*B*) Dot plot showing geomean expression of FACS surface markers on Ki67$^+$ or Ki67$^-$ memory CD4$^+$ T cells in patients with IBD before therapy (n = 41). Expression normalized to naïve CD4$^+$ T cells. (*C*) Percentage of indicated surface marker expression within Ki67$^+$ and Ki67$^-$ memory CD4$^+$ T cells in patients with IBD. n = 29–44. Mann-Whitney test. (*D*) Percentage of indicated transcription factor expression within Ki67$^+$ and Ki67$^-$ memory CD4$^+$ T cells in patients with IBD. n = 28–40. (*E*) Percentage of IL-17A and interferon (IFN)-gamma expression within Ki67$^+$ and Ki67$^-$ memory CD4$^+$ T cells in patients with IBD. n = 20. (*F*) Expression of the indicated transcription factors within Ki67$^+$ and Ki67$^-$ memory CD4$^+$ T cells in vedolizumab responders (R) vs nonresponders (NR). n = 18–32. Mann-Whitney test.

gluten-specific and *Salmonella*-specific circulating CD4$^+$ T cells from patients with celiac disease[37] or healthy volunteers challenged with *Salmonella enterica*[38] showed a comparable phenotype to the cycling effector cells in patients with IBD. This similarity suggests that the observed CD4$^+$ T cells in patients with IBD, particularly vedolizumab nonresponders, could be cycling, activated microbiota-specific CD4$^+$ T cells, which are known to be abundant in patients with IBD.[39]

The observed circulating Ki67$^+$ memory CD4$^+$ T cells expressed homing receptors (CXCR3, CCR6, and GPR15) and adhesion molecules (integrins $\alpha4\beta1$ and $\alpha4\beta7$), which enable migration to the inflamed mucosal tissue.[40,41] Indeed, a significant fraction of Ki67$^+$ memory CD4 T cells expressed integrin $\alpha4\beta1$ and, to a lesser extent, integrin $\alpha4\beta7$, suggesting that they might not be sufficiently targeted by anti-$\alpha4\beta7$ integrin treatment. Integrin $\alpha4\beta1$ binds to VCAM-1 and integrin $\alpha4\beta7$ interacts with MadCAM-1, and both VCAM-1 and MAdCAM-1 are up-regulated in the intestinal tissue of patients with IBD.[42,43] However, vedolizumab does not influence the interaction between integrin $\alpha4\beta1$ and VCAM.[12] Initial efforts to target cell migration to intestinal tissue using a monoclonal antibody targeting $\alpha4$ integrin, natalizumab, which blocks both $\alpha4\beta1$ and $\alpha4\beta7$ integrins, significantly reduced intestinal inflammation, but at the cost of elevated risk of fatal progressive multifocal leukoencephalopathy.[44–47] A small molecule oral integrin $\alpha4$ antagonist (AJM300) was well tolerated and induced a clinical response in patients with moderately active UC without increased risk of progressive multifocal leukoencephalopathy, which might be useful in patients with increased cycling circulating memory CD4$^+$ T cells.[48] However, the pathogenic role of these cells and their mechanism of targeting remain unclear and need further investigation.

This study has several limitations. First, our sample size is small, comprising mostly patients with UC. Second, our study does not include the analysis of matched mucosal samples, limiting our understanding of the origin and characteristics of the observed increase in proliferating memory CD4$^+$ T cells. Third, our novel findings of a predictive signature warrant additional investigations to elucidate underlying mechanisms. It is important to note that not all patients were profiled on all platforms, which may introduce bias or incomplete data representation. Nevertheless, our study expansively characterizes the circulating immune cell landscape using multimodal profiling, identifies alterations induced by vedolizumab treatment, and classifies therapy response. Validation of the predictive value of Ki67$^+$ memory CD4$^+$ T cells using a custom flow cytometry panel supported the robustness of our technical approach. However, a multicentric prospective study is needed to validate our signature in more heterogeneous patient populations. This will pave the way for developing a peripheral blood assay for precision therapy after validation in future multicentric studies.

Our study provides a comprehensive framework for assessing therapy response and understanding the mechanisms underlying resistance in chronic immune-mediated inflammatory diseases, such as IBD. Identifying personalized treatment strategies based on individual patient characteristics, as exemplified by our "stratify to target" approach, can significantly improve IBD management.

## Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at https://doi.org/10.1053/j.gastro.2024.09.021.

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**Data Availability**

The data, analytical methods, and study materials will be made available to other researchers upon publication. Please refer to the Materials and Methods section for details.