

Circulating $\alpha 4\beta 7^+$ Memory T Cells in Pediatric IBD Patients Express a Polyclonal T Cell Receptor Repertoire

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Background: The integrin $\alpha 4\beta 7$ is highly expressed on activated T cells and is thought to direct homing of lymphocytes to the intestine. Since ulcerative colitis (UC) and Crohn's disease (CD) are characterized by mucosal oligoclonal T cells' expansion, we aimed to assess whether similar repertoire features are identified in circulating gut-specific memory T cells.

Methods: Memory CD3⁺ T cells were isolated from blood samples of control subjects and patients with active UC or CD and then FACS-sorted into $\alpha 4\beta 7^+$ and $\alpha 4\beta 7^-$ populations. DNA was extracted from each subset and subjected to next-generation sequencing of the TCR β . Different repertoire characteristics were compared between $\alpha 4\beta 7^+$ and $\alpha 4\beta 7^-$ subsets for each subject, and between groups.

Results: The percentages of memory T cells and $\alpha 4\beta 7^+$ cells were comparable between groups. $\alpha 4\beta 7^+$ memory T cells displayed a polyclonal distribution, in control subjects and in UC or CD patients, with similar indices of diversity. Strikingly, the clonal overlap between $\alpha 4\beta 7^+$ and $\alpha 4\beta 7^-$ T cells for each subject in all three groups was high, ranging between 20%–50%. We were unable to identify shared T cell clones that were specific to one of the groups.

Conclusion: $\alpha 4\beta 7^+$ memory T cells exhibited a polyclonal repertoire in both control subjects and patients with active inflammatory bowel disease, with high rates of overlap with $\alpha 4\beta 7^-$ memory T cells. Our study, along with additional recent reports, may suggest that the suppression of intestinal inflammation by vedolizumab is independent of the drug's effect on T cell migration to the gut.

Keywords: IBD, TCR, integrin, $\alpha 4\beta 7$, T cells, immune repertoire

Introduction

Integrins are cell surface glycoprotein receptors that bind to adhesion molecules and mediate homing of leukocytes to peripheral sites such as the intestine.¹ More than 20 different integrins have been identified that vary according to their expression pattern and specificity of ligand binding. These processes are further strengthened by the expression of different chemokine receptors, such as CCR9, that stabilize the interaction between the immune cells and the vessel walls during the extravasation process in peripheral sites. Drugs that interfere with this process have been developed for patients with inflammatory bowel disease (IBD) or multiple sclerosis, based on selectivity of the integrin.^{2,3}

The $\alpha 4\beta 7$ integrin complex binds mucosal addressin cell adhesion molecule 1 (MAdCAM-1), expressed exclusively on intestinal endothelial cells.⁴ Ligation of $\alpha 4\beta 7$ and MAdCAM-1 leads to leukocyte extravasation into intestinal high

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endothelial venules and is therefore considered gut-selective. Understanding this process led to the development of vedolizumab, a monoclonal antibody targeting the $\alpha 4\beta 7$ integrin. Vedolizumab has been shown to be effective for induction and maintenance of remission in Crohn's disease (CD) and ulcerative colitis (UC) in multiple studies.^{5–9} Its main mechanism of action has been considered to be the blockade of activated T cell migration to the gut, as $\alpha 4\beta 7$ is upregulated on activated cells.⁴ However, emerging data have challenged this dogma by suggesting that other immune cells, and specifically innate immune populations, also express $\alpha 4\beta 7$,^{10–13} and question whether vedolizumab alleviates intestinal inflammation solely by blocking migration of T cells.

The adaptive immune arm contains trillions of different T cell clones. The marked diversity in T cell receptors (TCRs) is formed following a complex rearrangement process of different gene segments (VDJ recombination).¹⁴ There are four types of TCR protein monomers: α , β , γ and δ ; more than 95% of T cells express α/β chains, encoded by genes in the *TCRA* and *TCRB* loci. The antigenic specificity of T cells occurs via generation and rearrangement that involves recombination of variable (V β), diversity (D β) and joining (J β) genes, accompanied by deletion and insertion of random nucleotides, generating trillions of unique TCRs. Each TCR recognizes a unique antigen, and ligation can trigger proliferation, differentiation and/or cytokine secretion. Next-generation sequencing (NGS) platforms, developed in the last decade, facilitate detailed assessment of TCR repertoire patterns at the nucleotide or amino acid level.¹⁵ A restricted TCR (also referred to as oligoclonal expansion) was demonstrated in various autoimmune disorders including multiple sclerosis,¹⁶ rheumatoid arthritis¹⁷ and psoriasis.¹⁸ Few NGS repertoire studies in IBD revealed oligoclonality in intestinal samples of patients with active CD and UC.^{19–23} We were able to show that newly-diagnosed, pediatric UC patients have marked alterations in *TCRB* repertoire patterns in inflamed rectum, compared with controls, characterized by oligoclonal expansion and decreased diversity.²⁴

There are no data on TCR repertoire patterns of circulating memory T cells, and specifically those subsets with upregulated $\alpha 4\beta 7$ expression, among IBD patients. Since T cells are important in mediating the mucosal inflammatory response in IBD, one could expect that cells expressing $\alpha 4\beta 7$ would include a high percentage of disease-associated clonotypes. We aimed to examine repertoire features of $\alpha 4\beta 7^+$ memory T cells in IBD patients vs

control subjects, and specifically compare these features between $\alpha 4\beta 7^+$ and $\alpha 4\beta 7^-$ populations.

Methods

Sorting and DNA Isolation

The study was conducted in accordance with the Declaration of Helsinki and approved by the local ethics committee of Sheba Medical Center. Informed written consent was obtained from parents of participating subjects before experiments were conducted. Blood samples were obtained in EDTA-containing tubes from control subjects and patients with UC or CD. Disease activity in IBD patients was determined based on the pediatric ulcerative colitis activity index (PUCAI), the pediatric Crohn's disease activity index (pCAI) or endoscopic features. Samples were subjected to lymphoprep gradient centrifugation (Stemcell, MA, USA) for isolation of mononuclear cells. Next, negative selection of CD3⁺ cells was conducted using MACS beads (Miltenyi Biotec, CA, USA). Cells were stained with CD3-FITC, CD45RO-APC, (both from Miltenyi Biotec) and $\alpha 4\beta 7$ -PE (using vedolizumab as the antibody). Conjugation of vedolizumab to R-Phycoerythrin (R-PE) was performed using the SiteClick™ antibody labeling kit (Thermo Scientific, MA, USA). Antibody carbohydrate domain modification, azide attachment to the antibody, and conjugation with DIBO-modified label were performed according to manufacturer's instructions. CD3⁺ cells were subjected to FACS sorting into two sub-populations: memory T cells (CD3⁺CD45RO⁺) that are either $\alpha 4\beta 7^+$ or $\alpha 4\beta 7^-$ ([Supplementary Figure 1](#)). Purity was >95%. Genomic DNA was extracted from blood using a commercially available kit (Wizard kit, Promega, WA, USA), according to the manufacturer's instructions.

Next-Generation Sequencing

Up to 2 μ g DNA were used for TRB library generation of various V and J gene segments of the rearranged complementary determining region (CDR3 β , ImmunoSeq TRB Survey Service, Adaptive Biotechnologies, Seattle, USA). To ensure equal depth of sequencing, we used the Survey level (up to 500,000 reads per sample). The resulting libraries were subjected to high-throughput sequencing using Illumina technology. Samples were sequenced in two separate batches: first, controls and patients with UC and second, patients with CD. The number of sequences in the second run on the illumina was significantly lower

compared to the first run ([Supplementary Table 1](#)), but was still high, enabling a thorough analysis of immune repertoire patterns in the CD group as well.

TCR β Repertoire Analysis

ImmunoSeq software was used for determination of diversity parameters and overlapping clones. Clonality was calculated as 1-normalized Shannon's entropy. This measures how evenly receptor sequences are distributed among a set of T cells, with values ranging from 0 to 1. Values near 1 represent samples with one or a few predominant clones (monoclonal or oligoclonal samples) dominating the repertoire. Clonality values near 0 represent polyclonal samples. Simpson's D is the sum over all observed rearrangements of the square fractional abundances of each rearrangement. Shannon's H, which measures the overall diversity in a given population, and takes into account the number of unique sequences (richness of the repertoire) and how evenly the sequences are distributed, was calculated using the following formula:

$$\text{Shannon's } H = - \sum_{i=1}^R p_i \ln p_i$$

R = Total templates

i = Unique rearrangements

p_i = Proportion of the total sequences belonging to the "i"th unique rearrangement.

Graphical presentation of the repertoire was presented using hierarchical tree maps using the Treemap software (www.treemap.com).

Since the number of total templates differed between the groups, and to enable a valid comparison of different repertoire characteristics, we used the 10,000 most frequent unique clones for all analyses except for overlap between $\alpha 4\beta 7^+$ and $\alpha 4\beta 7^-$ populations for each subject.

Statistical Analysis

Values are expressed as mean $\pm$ standard error of the mean (SEM). Unpaired Student's *t*-test was used to test for statistical significance. Significance was determined if P value was ≤ 0.05 . For differential V-gene, and J-family usage, Bonferroni's correction for multiple comparisons was performed.

Results

Study Population

Blood samples were obtained from 5 control subjects, 6 patients with UC and 5 patients with CD ([Table 1](#)). Control subjects were patients referred for endoscopic evaluation of abdominal pain or diarrhea, that had

a normal macroscopic and histologic esophagogastroduodenoscopy or colonoscopy, without past or present history of IBD or other immune-mediated disorder (eg, celiac, diabetes, etc.). Among patients with UC, three patients had mild disease (based on the PUCAI score) and two experienced moderate disease activity. An additional patient was in clinical remission, but colonoscopy demonstrated moderate colitis (Mayo score 2). In the cohort of patients with CD, three patients had moderate disease activity (based on the pCDAI score), two had mild activity and one was in clinical remission, but with endoscopic and histologic evidence of inflammation of the ileocecal region, with large ulcers and cobblestoning. The median age of the subjects in the control group was 15.3 years (interquartile range [IQR] 13.5–16.4), compared with 16.8 years (IQR 15.0–17.9) and 15.8 years (IQR 15.1–17.5) in the UC and CD groups, respectively.

Immunophenotyping of Memory T Cells

We first analyzed the frequency of different immune populations in the studied groups. The frequency of $CD3^+CD45RO^+$ memory T cells was comparable between groups ($43.8 \pm 5.5\%$, $32.2 \pm 5.4\%$ and $47.0 \pm 6.0\%$ among control, UC and CD groups, respectively, [Figure 1A](#)). Moreover, the frequency of $\alpha 4\beta 7^+$ cells among memory T cells was also similar ($33.6 \pm 7.0\%$, $36.0 \pm 7.2\%$ and $37.4 \pm 4.1\%$, among control, UC and CD groups, respectively, [Figure 1B](#)).

Assessment of TCR β Repertoire Features

In order to characterize the landscape of memory T cells, NGS of the TCR β region was performed on $\alpha 4\beta 7^+$ and $\alpha 4\beta 7^-$ memory T cells. The mean number of total templates and unique templates in each of the groups is presented in [Supplementary Table 1](#). We first conducted treemap analysis of the most frequent 10,000 clones. In these studies, each square represents a unique clone and the size indicates how frequent it is. As can be seen in representative images in [Figure 2A](#), a polyclonal distribution of T cells was observed in $\alpha 4\beta 7^-$ and $\alpha 4\beta 7^+$ memory T cells, in control subjects, patients with active UC and CD. To further characterize the immune repertoire in these populations, various indices of diversity were calculated. The cumulative percent of the top 100 most frequent clones was comparable between $\alpha 4\beta 7^-$ and $\alpha 4\beta 7^+$ populations in all studied groups ([Figure 2B](#)). Moreover, Shannon's H, Simpson's D and clonality were also similar between sub-populations in controls, UC and CD patients, and also comparable between the groups ([Figure 2C–E](#)). These findings indicate that the TCR β

Table I Demographic and Clinical Phenotype of Studied Patients

		Gender	Current Age (yrs)	Age at Diagnosis	PUCAI/PCADI	Phenotype	Medication
Control	C1	M	16.0				None
	C2	M	15.3				None
	C3	M	16.8				None
	C4	F	12.5				None
	C5	F	14.5				None
UC	UC1	M	17.9	15.4	25	E4	Mesalazine
	UC2	M	15.8	13.7	20	E1	Prednisone, azathioprine
	UC3	F	17.8	12.6	15	E1	Mesalazine
	UC4	F	15.5	15.5	40	E2	None
	UC5	M	17.9	17.6	0*	E2	Mesalazine
	UC6	M	13.5	13.5	40	E4	None
CD	CD1	M	14.8	12.2	37.5	L2 [‡]	Adalimumab
	CD2	M	15.8	13.1	17.5	L1	Ustekinumab
	CD3	F	15.4	5.5	32.5	L1	Adalimumab
	CD4	F	17.0	16.2	30	L1	Prednisone, azathioprine
	CD5	F	18.0	14.0	0 [#]	L1	Infliximab

Notes: *Patient in clinical remission but with endoscopic evidence of inflammation (Mayo 2). [#]Patient in clinical remission but with evidence of inflammation of ileocecal region, with large ulcers and cobblestoning. [‡]Patient with initial L1 phenotype that changed after ileocecal resection to L2. For patients receiving biologics blood was drawn before receiving the scheduled dose of the drug.
Abbreviations: M, male; F, female; UC, ulcerative colitis; CD, Crohn's disease; E1, proctitis; E2, left-sided colitis; E4, pancolitis; L1, ileocecal disease; L2, colonic disease; PUCAI, pediatric ulcerative colitis activity index; pCDAI, pediatric Crohn's disease activity index.

repertoire of $\alpha 4\beta 7^{+}$ memory T cells is polyclonal, similar to the $\alpha 4\beta 7^{-}$ population, and that comparable diversity features are identified in these immune subsets in controls vs patients with active UC or CD.

Identification of Overlapping Clones

We next assessed whether there were overlapping clones between $\alpha 4\beta 7^{-}$ and $\alpha 4\beta 7^{+}$ memory T cells in each subject, and whether specific clonotypes could be identified solely in

one of the groups. Strikingly, the degree of clonal sharing between $\alpha 4\beta 7^{-}$ and $\alpha 4\beta 7^{+}$ sub-populations in each of the groups was high, ranging from 24%–50%, 29%–46% and 24%–50% in the control, UC and CD groups, respectively (Figure 3A and B). Comparison of specific clonotypes showed that among the 10 most frequent clones in each immune subset, for each subject, the mean number of shared clones was 2.4, 3.7 and 2.6 in the control, UC and CD groups, respectively (Supplementary Table 2).

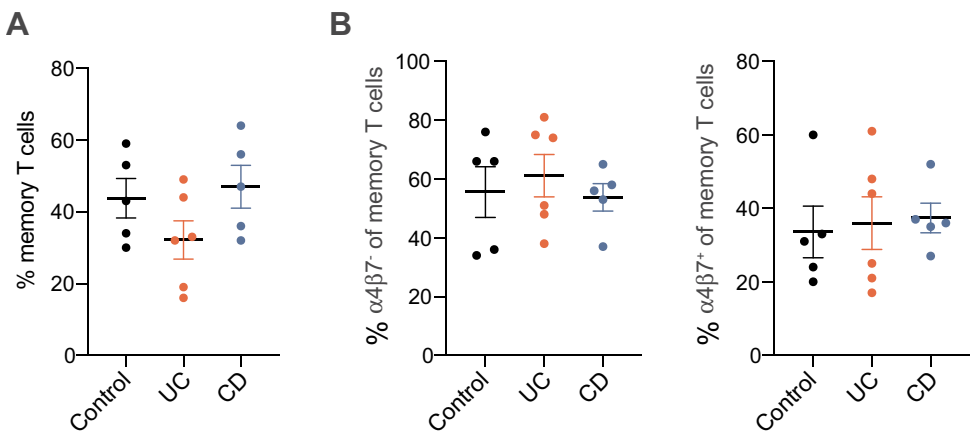


Figure 1 Comparable immune cell frequencies among studied patients and controls. Figure displays frequency of (A) memory CD3⁺CD45RO⁺ T cells and (B) $\alpha 4\beta 7^{+}$ and $\alpha 4\beta 7^{-}$ memory T cells in control subjects and patients with CD or UC.

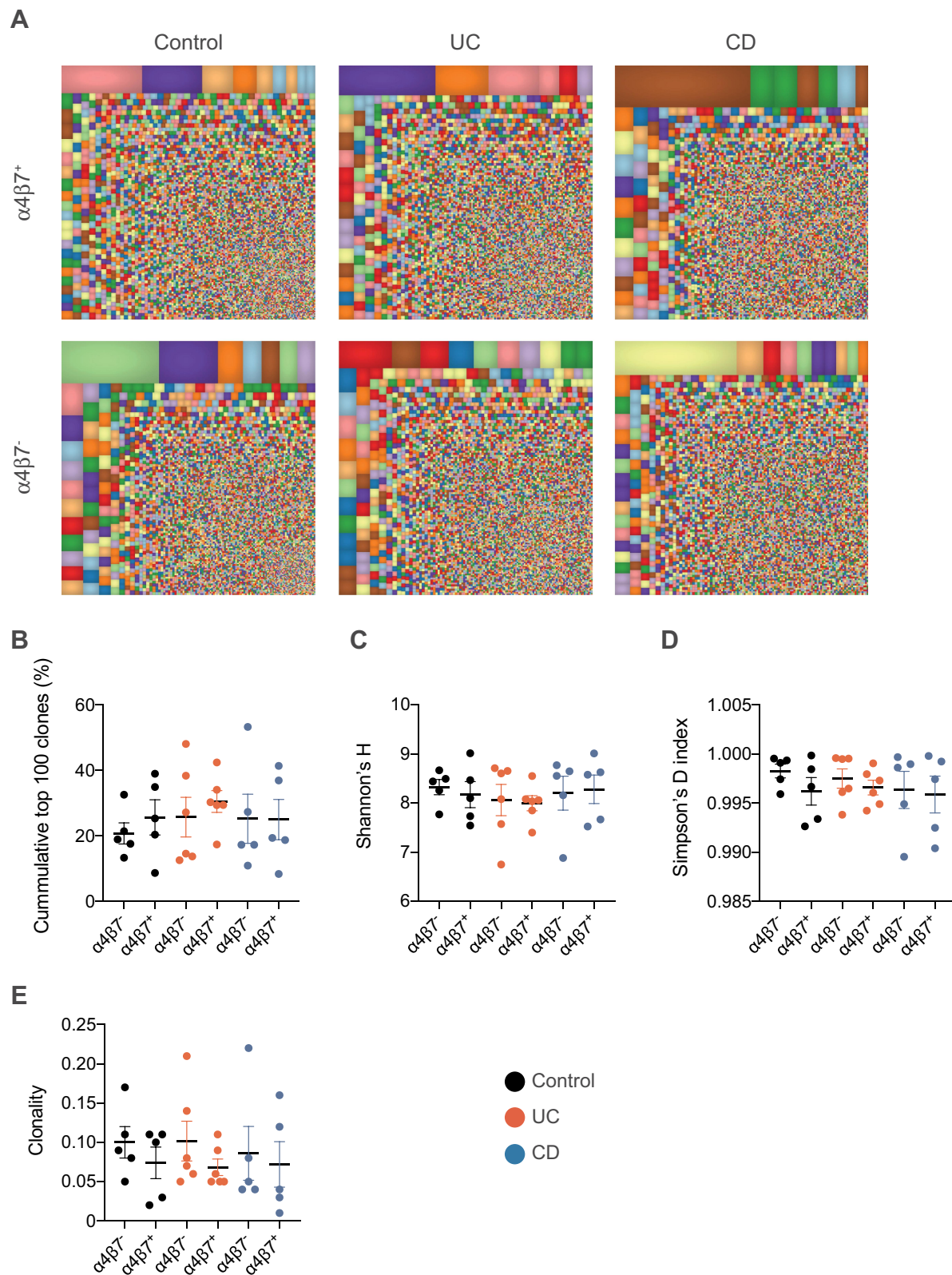


Figure 2 Polyclonal TCR repertoire characterizes circulating $\alpha 4\beta 7^+$ memory T cells. **(A)** Representative treemap images showing repertoire landscape in a control subject and patients with UC and CD. **(B)** Cumulative percentage of top 100 clones followed by representation of markers of diversity, including **(C)** Shannon's H, **(D)** Simpson's D and **(E)** clonality.

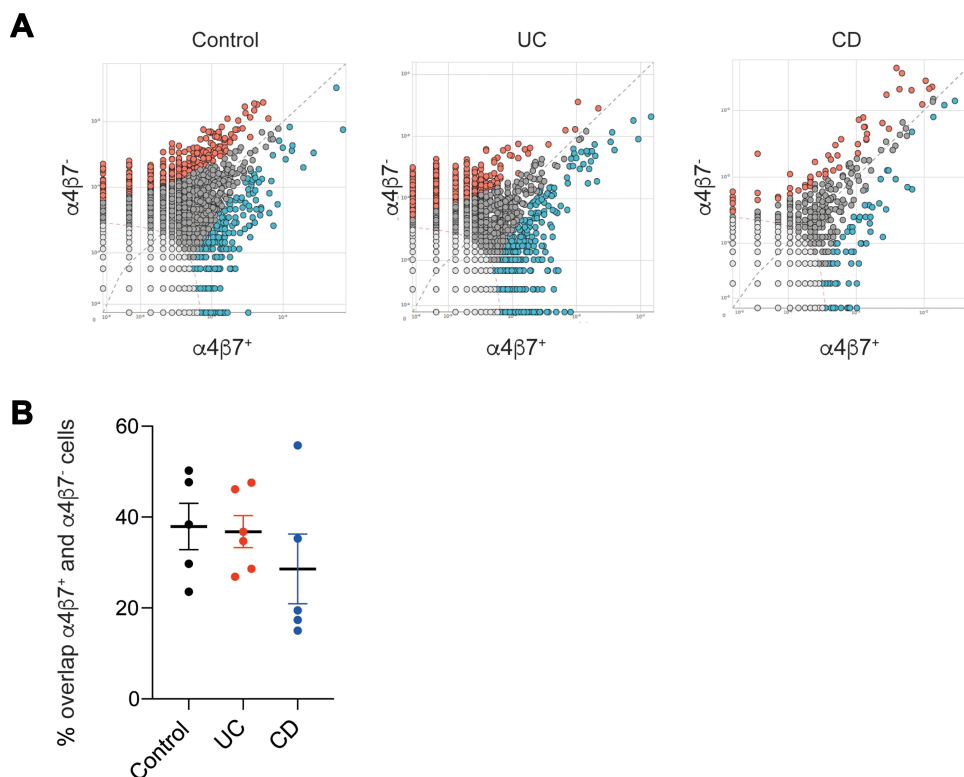


Figure 3 High degree of clonal overlap between $\alpha 4\beta 7^+$ and $\alpha 4\beta 7^-$ memory T cells. (A) Representative pair-wise scatters between $\alpha 4\beta 7^+$ and $\alpha 4\beta 7^-$ memory T cells in a control subject, and patients with UC or CD, followed by (B) cumulative analysis.

We next analyzed whether we could identify $\alpha 4\beta 7^-$ or $\alpha 4\beta 7^+$ memory T clones that were unique to each group. However, such specific clones that characterize solely one group could not be identified (data not shown). In addition, analysis of TRBV and TRBJ gene usage from each group showed similar frequencies, in both $\alpha 4\beta 7^-$ or $\alpha 4\beta 7^+$ subpopulations (Figure 4), except for TCRBV25 which was significantly lower in both UC and CD patients compared with controls, similar to previous observations.²⁵

Discussion

Next-generation sequencing is a powerful tool to study adaptive immune function in different diseases. Using this methodology, we and others have demonstrated oligoclonal expansion of T cells in the inflamed gut of patients with IBD.^{19–24} Since these T cells migrate to the gut, one could also expect to identify specific clonotypes in the blood of these patients, at least in those cells expressing $\alpha 4\beta 7$. Nevertheless, we showed a polyclonal distribution of T cell clonotypes in circulating $\alpha 4\beta 7^+$ and $\alpha 4\beta 7^-$ memory T cells in control subjects and in patients with active IBD. Moreover, we showed that the overlap between

$\alpha 4\beta 7^+$ and $\alpha 4\beta 7^-$ subsets was high, ranging between 20%–50%, highlighting that expression of this integrin is not confined to specific clones that are thought to be gut-specific.

Numerous studies have demonstrated that vedolizumab is effective in the treatment of patients with active IBD.^{5–9} Additional trials have also shown efficacy of new gut-specific anti-integrins in both UC and CD, such as etrolizumab,²⁶ targeting $\beta 7$, abrilumab,²⁷ targeting $\alpha 4\beta 7$ and AJM300,²⁸ targeting $\alpha 4$. Since $\alpha 4\beta 7$ was shown to be upregulated in memory T cells, and binds MADCAM1 expressed exclusively on intestinal endothelial cells,⁴ anti- $\alpha 4\beta 7$ drugs are thought to exert their anti-inflammatory effects through blocking migration of T cells to the gut,⁴ which could explain the relatively long duration needed to demonstrate clinical and endoscopic efficacy. However, data from different mouse and human studies in recent years suggest that targeting $\alpha 4\beta 7$ or $\beta 7$ may affect other immune populations beside T cells. Villablanca et al reported that $\beta 7$ expression on bone marrow precursors was required to facilitate reconstitution of small bowel tolerogenic retinoic acid-producing dendritic cells (DCs)

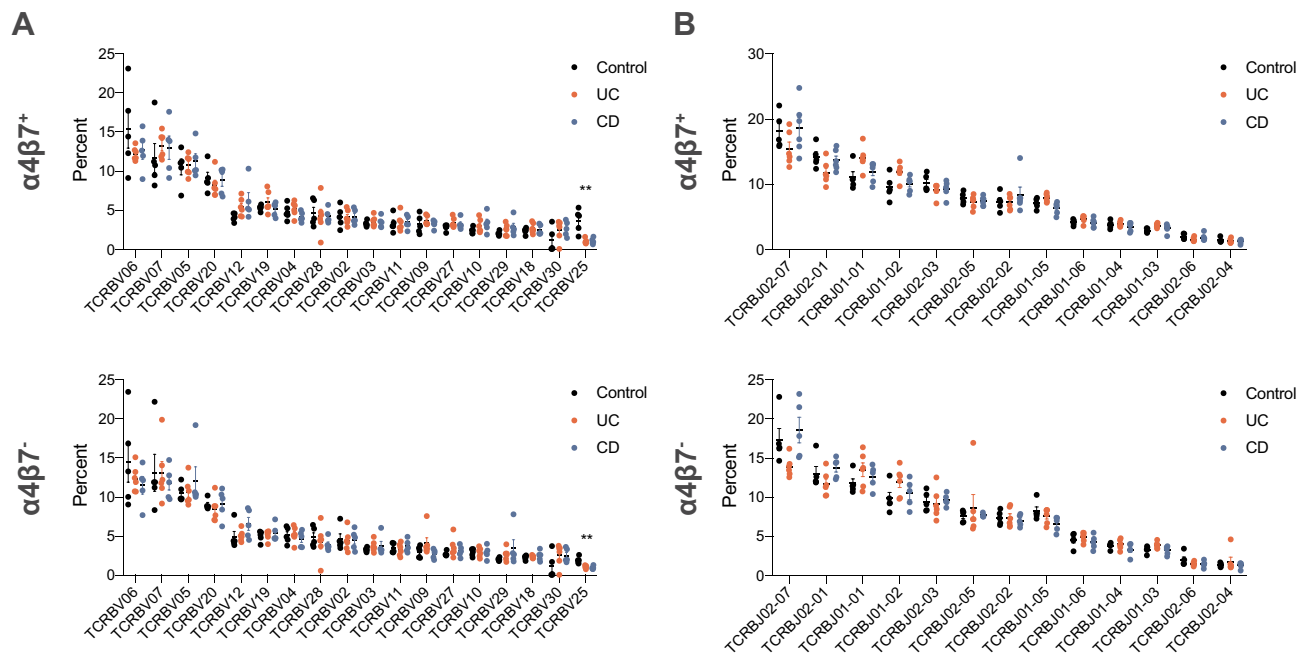


Figure 4 TRB gene usage in control subjects and IBD patients. Comparison of usage of the (A) TRBV genes and (B) TRBJ family genes in $\alpha 4\beta 7^+$ (top panel) and $\alpha 4\beta 7^-$ (bottom panel) memory T cells in control subjects, patients with UC and those with CD. For TRBV figures depict only genes with >1% frequency. **P value=0.002.

and macrophages.¹⁰ In addition, Schippers et al identified that $\beta 7$ expression on inflammatory monocytes was required for their migration to the gut, in mice.¹¹ Drugs targeting $\alpha 4\beta 7$ or $\beta 7$ may affect migration of these innate subsets as well.

Two additional studies shed more light on the importance of $\alpha 4\beta 7$ expression on monocytes in IBD. Zeissig et al compared systemic and mucosal immune profiles of 18 IBD patients treated with vedolizumab vs 20 patients treated with anti-TNF α .¹² While they were not able to show differences in the frequency or phenotype of mucosal T cells in patients treated with vedolizumab, before and after induction timeframe, they did identify marked changes in macrophage abundance, phenotype and pattern recognition receptor expression.¹² Moreover, the intestinal TCR α and TCR β repertoires pre- and post-vedolizumab therapy were comparable, even after 14 weeks. Another study showed that $\alpha 4\beta 7$ was expressed on CD14⁺CD16^{high} non-classical monocytes and mediated their migration to the gut, where they develop into CD163⁺ wound healing macrophages.¹³ Using *Rag1*^{-/-} mice they showed that anti- $\alpha 4\beta 7$ inhibited colonic wound healing in a T cell-independent manner.²¹

Overall, the previously mentioned studies suggest that vedolizumab may affect gut homing of multiple immune cell populations, and that its effects on innate cells might

be more important than previously appreciated. Our findings also question the importance of $\alpha 4\beta 7$ for guiding T cell migration to the gut, since a high degree of overlap was identified between $\alpha 4\beta 7^+$ and $\alpha 4\beta 7^-$ memory T cells. Interestingly, the IBD group in Sheba Medical Center showed that vedolizumab therapy completely blocked $\alpha 4\beta 7$ expression on circulating T cells, independent of patient's response status or vedolizumab serum levels,²⁹ suggesting that the integrin blockade on T cells may not be sufficient on its own for patient's response to treatment. It is possible that surface expression of $\alpha 4\beta 7$ on T cells is completely blocked by vedolizumab, as shown by that group,²⁹ but there are still free molecules on innate cells and therefore full treatment efficacy is not achieved. Another alternative explanation is that vedolizumab may affect the function of mucosal T cells directly, irrespective of its effect on cell migration.

The overlap between circulating $\alpha 4\beta 7^+$ and $\alpha 4\beta 7^-$ memory T cells can be explained in several ways. It is possible that different factors up- or down-regulate $\alpha 4\beta 7$'s expression in the same cell and that levels of expression vary. Moreover, migration of T cells is influenced by expression of different chemokine receptors,⁴ which were not examined here. Finally, additional integrins might be important as well for gut-specific migration, including $\alpha E\beta 7$.³⁰

Our study has several limitations, including the small sample size in each of the groups and relatively mild clinical disease activity, especially in the UC group. However, the data of polyclonal repertoire features and high degree of clonal overlap were consistent in all subjects from the three groups. In addition, different therapies and especially immunosuppressive regimens can affect repertoire features by inhibiting proliferation of T cells. In our UC cohort one out of six subjects was receiving prednisone and azathioprine, while the rest were on mesalazine or treatment naïve. Among the patients with CD, all were on medications, including prednisone and azathioprine or biologics. It is possible that these drugs affected repertoire features, and further studies among treatment-naïve patients should address this point. Finally, we did not sequence the TCR α region, although it is well established that the most variable region is the CDR3 β of the TCR β . Nevertheless, this is the first study looking at immune repertoire profiles in specific T cell subsets in IBD patients. We used vedolizumab-conjugated antibody to sort $\alpha 4\beta 7^+$ cells, and therefore believe the identity of the subpopulations was correct. Moreover, our study enabled a comparison between $\alpha 4\beta 7^+$ and $\alpha 4\beta 7^-$ populations, with similar results showing high degree of clonal overlap in all groups. Further studies should assess whether similar profiles are identified in mucosal $\alpha 4\beta 7^+$ and $\alpha 4\beta 7^-$ memory T cells, and compare them to circulating T cells.

In conclusion, we showed a polyclonal distribution of $\alpha 4\beta 7^+$ memory T cells in IBD patients, similar to control subjects, along with a high degree of clonal overlap between $\alpha 4\beta 7^+$ and $\alpha 4\beta 7^-$ memory T cells. While it is clear that vedolizumab is an effective drug for patients with active IBD, its mechanism of action might be broader than blocking T cell migration to the gut. Additional studies are required to characterize the dynamics of various integrins on specific immune populations in the intestine (small vs large bowel) and blood, and define these expression profiles in steady state, during active inflammation and in mucosal healing.

Disclosure

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