Gut-liver interactions in primary sclerosing cholangitis and inflammatory bowel disease

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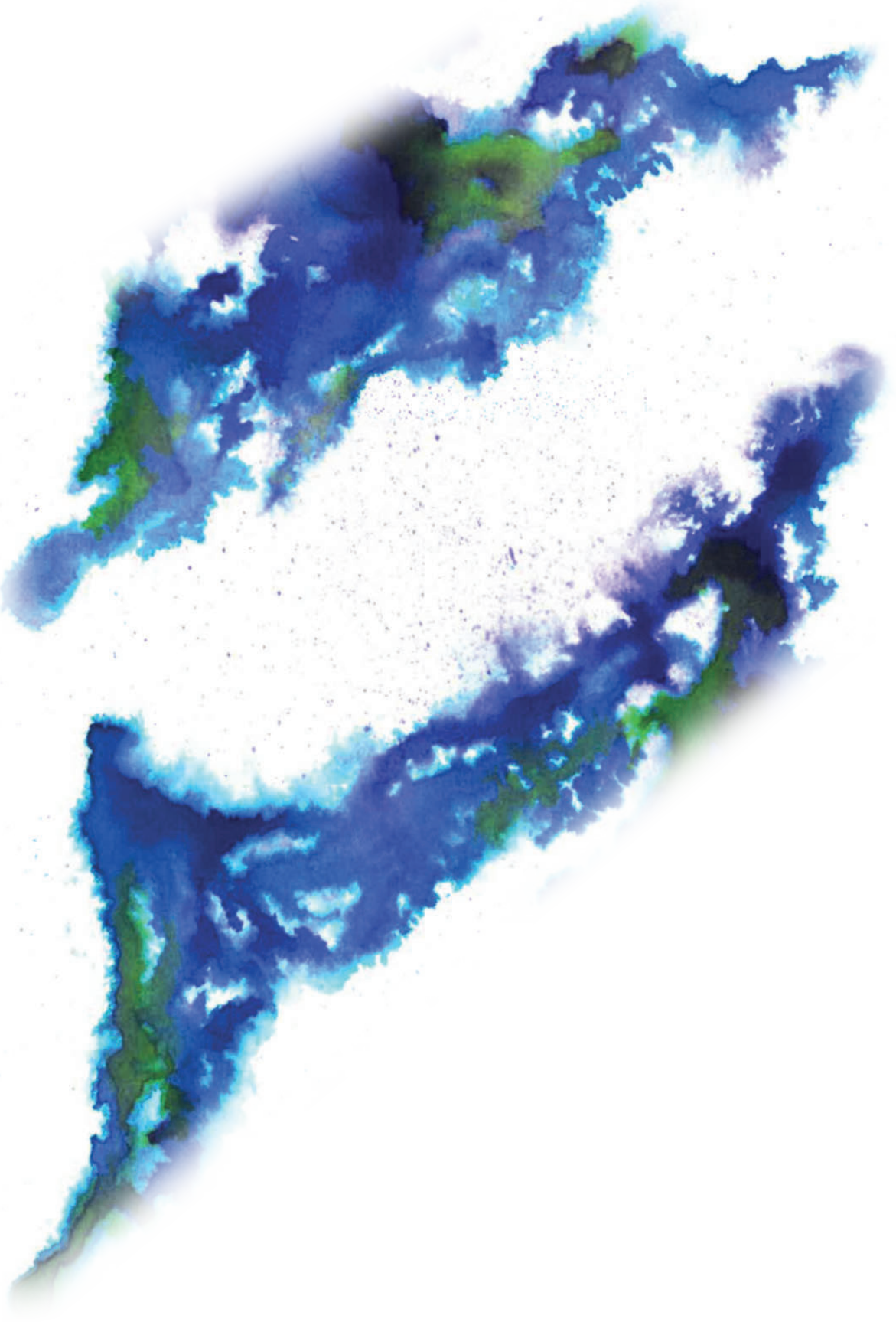

CHAPTER 4

Expression of MAdCAM-1 and gut-homing T-cells in inflamed pouch mucosa

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Abstract

Background and aims

Pouchitis is a common complication following formation of an ileal pouch-anal anastomosis (IPAA) after proctocolectomy for ulcerative colitis (UC). Gut-specific lymphocyte trafficking mechanisms have been identified as players in the pathogenesis of UC. In the present study, we aimed to characterize the presence of lymphocyte subsets expressing gut-homing molecules in pouches and peripheral blood of UC patients with and without pouchitis.

Methods

Biopsy samples and peripheral blood were collected from 29 patients with an IPAA (7 with active inflammation, 22 without inflammation). Expression of adhesion molecule MAdCAM-1 was assessed using immunohistochemistry, and flow cytometry was used to characterize expression of integrin α4β7, C-chemokine receptor 9 (CCR9) and CD103 on T-cell subsets.

Results

MAdCAM-1 expression was significantly increased in case of active inflammation in the pouch. T-cells expressing integrin α4β7 were abundant in the pouch mucosa, but the frequency of integrin α4β7 expressing T-cells was decreased on $CD4^+$ lymphocytes during inflammation. Co-expression of gut-homing markers CCR9 and α4β7 was more pronounced in biopsies compared with peripheral blood, but was not enhanced upon active inflammation.

Conclusions

Gut-homing T-cells are abundant in pouch mucosa, but the classic hypothesis that the chronic inflammatory state is maintained by an accumulation of α4β7-expressing effector T-cells is not supported by our data.

Key words: pouchitis, integrin alpha4beta7, MAdCAM-1

Introduction

A substantial proportion of ulcerative colitis (UC) patients will need a proctocolectomy with ileal pouch-anal anastomosis (IPAA) because of medically refractory disease or colorectal neoplasia development.[1,2] A common complication of IPAA is pouchitis, a nonspecific inflammation of the pouch, which occurs at least once in up to 50% of UC patients with IPAA, and in a chronic form in 5-30% of patients.[3-6] In contrast to this, the incidence of pouchitis in patients with an IPAA because of familial adenomatous polyposis (FAP) is much lower, ranging from 11 to19%.[5,7] This suggests that pouchitis in UC patients is attributable to an abnormal immune response, but the etiology of pouchitis is not well understood. This association with UC suggests that treatment with agents used in UC could be of benefit.

An important component of directing pro-inflammatory lymphocytes to the intestine is the attraction by integrins and chemokines. Naïve T-lymphocytes are primed towards a gut-specific phenotype in the mucosa associated lymphoid tissue. Dendritic cells can induce this gut-tropism by upregulating integrin α4β7 and CC-Chemokine receptor 9 (CCR9) on lymphocytes via the conversion of retinol to retinoic acid to act on RALDH nuclear receptors.[8,9] As such this enables lymphocytes to enter the gut lamina propria through binding to Mucosal Addressin Cell Adhesion Molecule-1 (MAdCAM-1) and C-C motif chemokine ligand 25 (CCL25). Although MAdCAM-1 is constitutively expressed on vascular endothelium in the gut, its expression is increased during active inflammation in the gut mucosa of patients with inflammatory bowel diseases (IBD).[10] Blocking this α4β7 integrin-mediated gut homing directly with vedolizumab or etrolizumab and more recently, via blockage of MAdCAM-1 with ontamalimab, has been proven effective as therapy in patients with IBD.[11-15] Vedolizumab has also been investigated for the treatment of pouchitis in several case series showing promising results, albeit with only small sample sizes.[16,17] However, the biological basis for this is unclear and whether gut-homing trafficking mechanisms are operational in IPAA remains to be investigated.

In this study, we aimed to delineate T-lymphocyte subsets expressing gut-homing molecules in pouches and peripheral blood of UC patients with an IPAA and to address whether there is an accumulation of these subsets in case of active inflammation. Additionally, we sought to investigate different key-players of lymphocyte trafficking mechanisms in pouches with and without active inflammation.

Materials and methods

Patients and samples

Mucosal biopsies from the pouch body and peripheral blood were obtained from a total of 29 patients that displayed either signs of pouchitis or not. Samples were acquired during surveillance or diagnostic endoscopy of the pouch. Patients could be enrolled when they had a history of IPAA for UC completed at least 3 months prior to screening, with an age

of 18 years or older. Baseline characteristics were obtained from the electronic patient file. Diagnosis of pouchitis was established using the pouchitis disease activity index (PDAI), which was calculated from 3 parameters including clinical symptoms, endoscopic findings, and histologic changes (Supplementary Table 1).[18,19] Groups were divided based on histological signs of active inflammation in the pertaining biopsy, reflected through histological PDAI score, as clinical and endoscopic signs of pouchitis do not always correlate with histological inflammation. A histological PDAI score of 2 or more (at least moderate neutrophilic granulocyte infiltration with crypt abscesses, or mild neutrophilic granulocyte infiltration and 0-25% ulcerations per low-power field) was used as a cut-off for inflammation ('inflamed'); a histological PDAI score of one or less was considered 'non-inflamed'. This resulted in a total of seven patients with and 22 patients without active inflammation. Clinical characteristics are depicted in Table 1. An infection with *Clostridium difficile* as an alternative cause for the inflammation of the pouch was ruled out on indication. The accredited Medical Ethics Committee at the Amsterdam UMC, University of Amsterdam approved the protocol (METC2016_333), all samples were collected with prior informed consent.

Flow cytometry

Directly after biopsy, two pouch biopsies were collected in complete medium (RPMI containing L-glutamine (Lonza, Allendale, NJ), heat-inactivated Fetal Bovine Serum (Serana, Pessin,Germany) and Penicillin/Streptomycin (Lonza, Verviers, Belgium)). Biopsies were mashed through a 70 µm cell strainer after digestion for 1 h at 37 ˚C in digestion medium (complete medium with 1mg/ml Collagenase D (Roche, Basel, Switzerland), 1 mg/ml Soybean Trypsin inhibitor (Invitrogen) and 50µg/ml DNAse I from bovine pancreas (Roche, Basel, Switzerland)). To avoid cell loss, no density-based separation was performed.

Peripheral blood was collected in either a Heparin (samples 1-16) or EDTA (samples 17-29) tube. Cells were stained after red blood cell lysis in lysis buffer (1.55M NH_4Cl, 0.1M $KHCO_3$, and 1mM EDTA in MiliQ) for 10-15 min.

Cells were characterized with a panel of 10 antibodies as described in Supplementary Table 2 (panel 1). After inclusion of the first 16 samples, the antibody panel was adjusted for additional cell characterization (Supplementary Table 2, panel 2). For detection of integrin α4β7, monoclonal antibody vedolizumab (Takeda, Tokyo, Japan) was directly labelled with a fluorochrome using the DyLight 650 Microscale Antibody Labeling Kit, according to manufacturer's protocol (Thermo Fisher Scientific). Samples 17-29 were stained with a live/dead marker (Fixable Red Dead Cell Stain Kit, Thermo Fisher Scientific/Life Technologies, Carlsbad, CA) for 30 min and subsequently incubated with the antibody panel for 30 min protected from light at 4˚C. All samples were fixed with 2% paraformaldehyde (PFA) before acquisition.

Absolute cell counts in biopsies (samples 17-29) were measured using Count Bright Absolute Counting Beads (Invitrogen) according to supplier's protocol. For samples 17-29, one EDTA tube was used to determine differential leukocyte count in peripheral blood.

Table 1. Patient characteristics

	Inflamed (n=7)	Non-inflamed (n=22)	P-value
Male [n (%)]	6 (86)	11 (50)	0.187
Age [median (IQR)]	45 (36-58)	43 (31-50)	0.280
Age at UC diagnosis [median (IQR)]	23 (15-42)	21 (17-27)	0.901
Years after IPAA surgery [median (IQR)]	12 (2-19)	9 (5-16)	0.980
PSC [n (%)]	2 (29)	6 (27)[#]	1.000
Deviating ileostomy [n (%)]	1 (14)	1 (5)	0.431
Medication use [n (%)]			
Antibiotics at time of endoscopy [n (%)]	3 (43)	7 (32)	0.665
Chronic antibiotics use [n (%)]	3 (43)	5 (23)	0.357
Biologicals	1 (14)*	3 (14)**	1.000
Mesalazine	1 (14)	1 (5)	0.431
Thiopurines	0 (0)	1 (5)	1.000
Other IBD medication[+]	1 (14)	1 (5)	0.431
Indication endoscopy [n (%)]			0.791
Complaints	5 (71)	16 (73)	
Surveillance	2 (29)	4 (18)	
Other[++]	0 (0)	2 (9)	
PDAI [median (IQR)]	11 (7-12)	5 (3-6)	0.004

[#] One patient post liver transplantation for PSC.

* Patient used Adalimumab.

** One patient used Adalimumab, one patient used Ustekinumab, and one patient used Golimumab for joint compaints.

[+] Beclomethason and mercaptopurine respectively.

[++] To address response to therapy, checkup 1 year after IPAA.

IQR, interquartile range; UC, ulcerative colitis; IPAA, ileal pouch-anal anastomosis; PSC, primary sclerosing cholangitis; IBD, Inflammatory Bowel Disease; PDAI, Pouch Disease Activity Index.

P-values were calculated with Fisher Exact test for binominal variables and Mann-Whitney U test for continuous variables.

All samples were analysed on a cell analyser (BD LSR Fortessa) with FACSDiva software (BD Biosciences, San Jose, CA). Data were analysed using FlowJo software (Treestar Inc., Ashland, OR). Single stained beads (Thermo Fisher Scientific) were used as compensation controls. Gates were set based on fluorescent minus one (FMO) stains. Gating strategies are depicted in Supplementary Figure 1. One patient was excluded from the analysis due to technical issues of the cell analyser.

Immunohistochemical staining

Fresh pouch biopsies were fixed in Tissue-Tek O.C.T. compound (Sakura Finetek, USA) and stored at -80˚C; 4,5 µm-thick slides were fixated in ice cold acetone for 10 min and washed with PBS. Endogenous peroxidase was blocked with Bloxall solution (Vectorlabs, Burlingame, CA) for 10 min, after which sections were incubated for 10 min with PBT (PBS

with BSA and Triton-X-100) to block non-specific binding sites. Diluted primary antibody (mouse anti-human MAdCAM-1 (clone 355G8, Invitrogen)) was added and incubated overnight at 4°C. BrightVision Poly-HRP from Immunologic (Duiven, the Netherlands) was used as secondary antibody and incubated for 1 h at room temperature. Staining was visualized with ImmPACT DAB peroxidase substrate (Vector Laboratories, USA) for 8 min, followed by counterstain with haematoxyilin. Human ileum was used as positive control for MAdCAM-1, and as a negative control, slides were incubated with secondary antibody only.

Fresh pouch biopsies were fixed in formalin and embedded in paraffin (FFPE) according to standard procedure. Haematoxylin and eosin (HE) staining was performed according to standard protocol at the pathology department of Amsterdam UMC, location AMC, The Netherlands.

Evaluation of histological staining

Histological PDAI score was assessed by a gastrointestinal pathologist (AM) on HE-stained FFPE slides. MAdCAM-1 staining was assessed based on the number of vessels with positively stained endothelial cells (at 200x magnification) divided by the area of the biopsy (in mm^2), yielding a density score. Biopsy area was determined using IntelliSite Pathology Suite (Philips, Amsterdam, The Netherlands). Two samples could not be analysed due to insufficient staining quality (one in each group).

Quantitative real-time polymerase chain reaction

Isolation of mRNA was performed using the Bioline ISOLATE II RNA mini kit (GC biotech B.V. Alphen a/d Rijn, The Netherlands) according to the manufacturer's instructions. RNA concentration was measured using the Nanodrop 1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). cDNA was synthesized using the Revertaid first strand cDNA synthesis kit (Fermentas, St. Leon-Rot, Germany). A quantitative polymerase chain reaction (qPCR) was performed using SensiFAST SYBR No-ROX (GC Biotech B.V.) on a BioRad (CFX96 real-time qPCR thermocycler) to analyze expression levels of *MAdCAM-1, CCL25 and CCR9.* For normalization, human reference genes ribosomal protein lateral stalk subunit P0 (*36B4)* and Glyceraldehyde 3-Phosphate Dehydrogenase (*GAPDH*) were selected after stability analysis in geNorm.[20] Primers (obtained from Qiagen or synthesized by Sigma) are listed in Supplementary Table 3.

Statistical Analysis

Patient characteristics are presented as median and interquartile range (IQR, 25^{th}-75^{th} percentile). Dichotomous variables are expressed as percentage (%) of the cohort. Differences were calculated with Chi-square test or Fisher's Exact test for categorical variables. Numerical data was compared using a Mann-Whitney U test or a Kruskal-Wallis test with Dunn's correction for multiple testing. Statistical analyses were performed using

SPSS statistical software for Windows version 26.0 (SPSS, Chicago, IL, USA) or GraphPad Prism 8. A p-value <0.05 was considered statistically significant.

Results

Clinical characteristics

Mucosal biopsies from seven patients with active inflammation and 22 patients without active inflammation were collected. Clinical characteristics are depicted in Table 1. Pouches were *in situ* for a median of 12 years in the inflamed group (IQR 2-19) and 9 years (IQR 5-16) in the non-inflamed group. There was no difference in medication use between the groups studied. The majority of patients (20/29, 69%) was diagnosed with chronic pouchitis, either antibiotic-refractory or antibiotic-dependent. In the inflamed group, the median total PDAI score was 11 (IQR 7-12) compared with 5 (IQR 3-6) in the non-inflamed group (p=0.004, Table 1, Supplementary Table 1).

MAdCAM-1 expression is increased in inflamed pouch mucosa

Protein expression of MAdCAM-1 was assessed in inflamed and non-inflamed pouches using immunohistochemistry (Fig. 1A). The density of MAdCAM-1 (number of MAdCAM-1 expressing endothelial vessels corrected for biopsy surface) was higher in inflamed mucosa compared to non-inflamed mucosa (p=0.040; Fig. 1B). This was confirmed by mRNA expression of *MAdCAM-1* between the two groups (p=0.017; Fig. 1C).

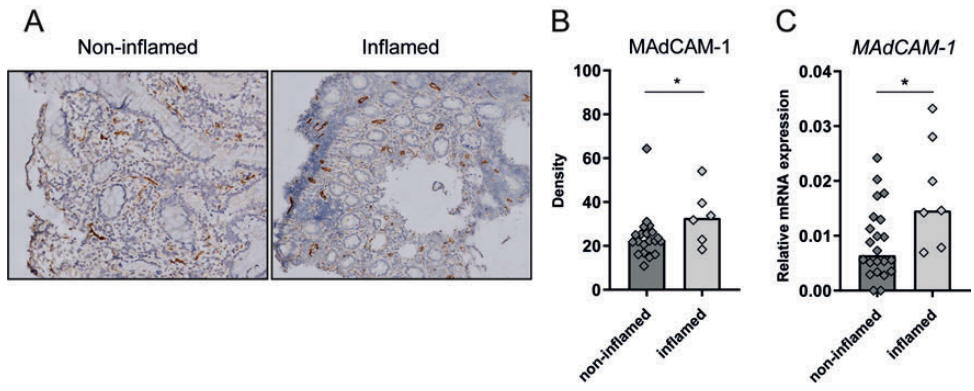

Figure 1. MAdCAM-1 expression is higher in inflamed pouch mucosa. A: Immunohistochemistry of MAdCAM-1 on frozen sections. Representative pictures of one pouch biopsy without inflammation (left) and with inflammation (right). Ileum tissue was used as a positive control for antibody specificity (not shown). B: Density of MAdCAM-1 staining in non-inflamed pouches (n=21) and inflamed pouch biopsies (n=6), based on histological PDAI score. Two biopsies could not be evaluated. Density was calculated as number of MAdCAM-1 expressing endothelial vessels corrected for biopsy surface. C: Relative mRNA expression of *MAdCAM-1* in non-inflamed pouches (n=22) and inflamed pouches (n=7). Data presented as median. A p-value <0.05 was considered statistically significant (*p<0.05, Mann-Whitney U test). PDAI, Pouch Disease Activity Index.

T-lymphocytes expressing α4β7 are abundant in pouch biopsies, but decreased in case of active inflammation

To address whether the ligand of MAdCAM-1, integrin α4β7, was also more abundant in inflamed pouches, tissue-infiltrating T-cells were analysed using flow cytometry. A previous study showed a higher abundance of $CD4^+$ T-cells in inflamed pouches, and suggested an increased pathological state of activation of $CD4^+$ T-cells in pouchitis.[21] Therefore, we first analysed the abundance of different T-cell subsets in the pouch mucosa. Irrespective of active inflammation, the majority of infiltrating T-cells were $CD4^+$ (median 53% $CD4^+$ and 31% $CD8^+$, $p<0.001$; Fig. 2A, Supplementary Fig. 1). This percentage of infiltrating $CD4^+$ lymphocytes was not increased upon inflammation, nor was the percentage of $CD8^+$ lymphocytes (Fig. 2B). Next, we characterized the proportion of memory, naïve, and regulatory T-cells (T-regs) within the T-helper ($CD4^+$) subset. As expected from intestinal mucosa, the majority of infiltrating $CD4^+$ lymphocytes displayed a memory phenotype ($CD45RO^+$, median 81%) [21,22], whereas around 2.3% showed a naïve ($CD45RO^-CCR7^+$) and 10% a regulatory ($CD127^{low}CD25^{high}$) phenotype (Fig. 2C). This trend was similar in inflamed and non-inflamed mucosa, although sample sizes were too small to draw firm conclusions (Fig. 2C).

Considering the higher expression of MAdCAM-1 in inflamed mucosa, we hypothesized that there would be a subsequent increase in cells expressing its ligand integrin α4β7 in case of pouchitis. Combining both antibodies for integrin α4β7 and integrin β7 in the first antibody panel induced an underestimation of the expression of both antibodies due to competitive binding (Supplementary Fig. 2). Therefore, only integrin α4β7 was included in the second antibody panel. Relative expression to the mean percentage of α4β7 positivity in each panel was used to correct for these panel differences. Inflamed and non-inflamed biopsies were evenly distributed between panels (4:11 for panel one and 2:11 for panel two, respectively). As previously described, integrin α4β7 was extensively expressed by $CD3^+$ T-cells in the intestinal mucosa (Fig. 3A).[23] There was no difference in relative expression of α4β7 on $CD3^+$ T-cells between inflamed and non-inflamed pouches ($p=0.178$; Fig. 3B). As there was a high abundance of $CD4^+$ cells in the pouch, we also determined α4β7 expression on $CD4^+$ and $CD8^+$ T-cells separately. Expression of integrin α4β7 was similar on $CD4^+$ and $CD8^+$ T-cells (Supplementary Fig. 3), but expression of α4β7 tended to be lower in inflamed mucosa compared with non-inflamed mucosa in all subsets (Fig. 3B). To address whether this decrease on $CD4^+$ T-cells was induced by differences in memory or regulatory cells, we further characterized the $CD4^+\alpha4\beta7^+$ subset. The majority displayed a memory phenotype, but there was no difference in subsets between inflamed and non-inflamed pouches (Fig. 3C). This suggests that there is an influx of mainly effector T-cells and not T-regs infiltrating the pouch mucosa. Of note, absolute cell counts did not differ between inflamed and non-inflamed pouches (Supplementary Fig. 4B).

The other dimerization partner of β7, integrin αE (CD103) is expressed predominantly by intraepithelial lymphocytes.[24,25] It is hypothesized that upon migration into the mucosa,

integrin α4 on T-cells is downregulated whereas αE is upregulated to promote retention in the gut.[26] Integrin αE was indeed highly expressed by CD8+ T-cells in the pouch biopsies, but its expression was significantly lower in case of active inflammation, whilst the total percentage of CD8+ T-cells stays the same (p=0.004; Fig. 3D).

The majority of patients had a chronic form of pouchitis, including multiple episodes of antibiotic treatment and intermitting inflammation of the pouch. Additional subgroup analysis was performed to address whether this decrease in memory CD4+α4β7+ T-cells was a direct effect of inflammation or a chronic event. Redistribution of patients without inflammation into a group without any previous episode of pouchitis and patients with chronic pouchitis without active inflammation showed that also in the chronic phase, there was a trend towards a decrease in expression of α4β7 (Fig. 3E). This was most prominent in the total CD3+ subset.

T-cells co-expressing integrin α4β7 and CCR9 are more abundant in pouch mucosa than in peripheral blood

Next, we sought to see whether this tendency towards less integrin α4β7 in inflamed pouches was attributable to a specific subgroup of gut-primed cells. Homing of effector T-cells to the small intestine requires both integrin α4β7 as well as chemokine CCR9. This chemokine is highly expressed on intestinal lymphocytes, showing regional

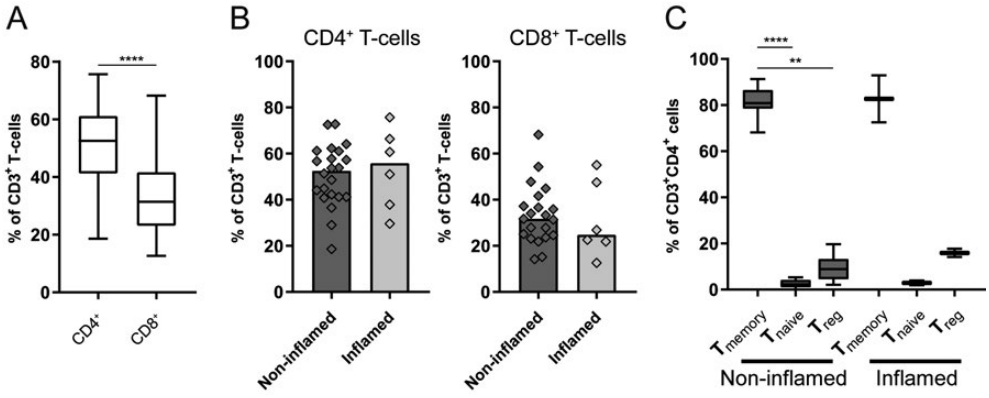

Figure 2. No differences in infiltrating lymphocytes in inflamed and non-inflamed pouches. Flow cytometry of pouch biopsies: differences between non-inflamed (PDAI histological subscore 0-1, dark grey) and inflamed (PDAI histological subscore ≥2, light grey) biopsies. A: Percentage of CD3+ T-cells expressing CD4 and CD8 in pouch biopsies (n=28). B: Difference in CD4+ T-cells (left) and CD8+ T-cells (right) between non-inflamed (n=22) and inflamed (n=6) biopsies. C: Distribution of memory (CD45RO+), naive (CD45RO-CCR7+), and regulatory (CD127^{low}CD25^{high}) phenotypes within CD4+ T-cells of non-inflamed (n=11) and inflamed (n=2) biopsies. Statistical testing was performed using the Mann-Whitney U test or Kruskal-Wallis with Dunn's correction for multiple testing. Data presented as median. A p-value <0.05 was considered statistically significant (**p<0.01, ****p<0.0001). PDAI, Pouch Disease Activity Index.

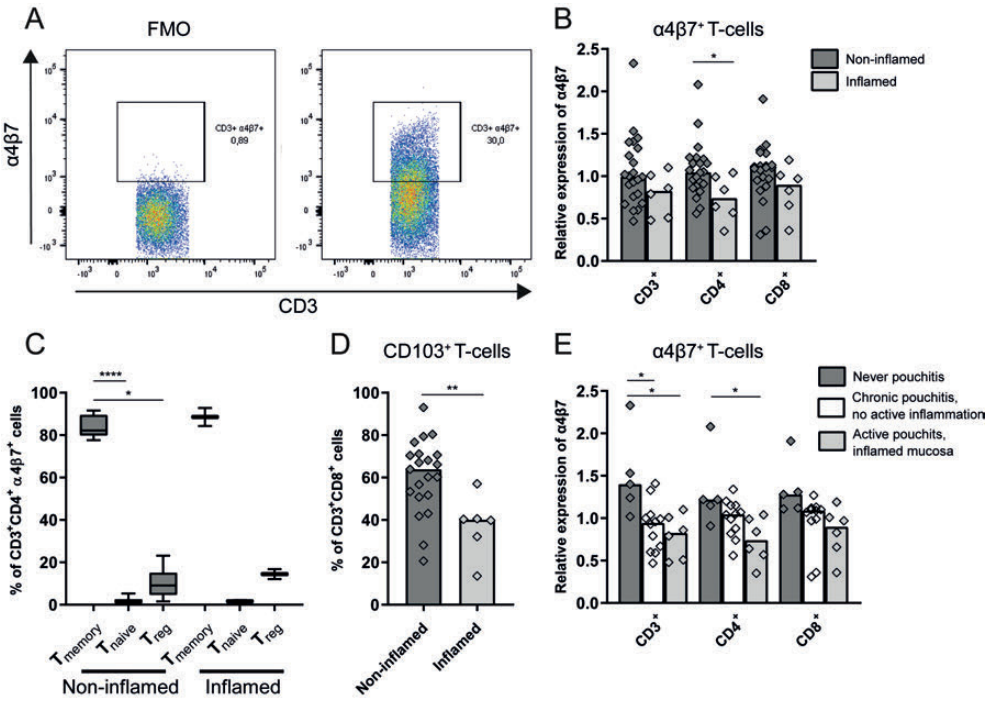

Figure 3. Integrin α4β7⁺ T-cells in inflamed and non-inflamed pouches. Flow cytometry of pouch biopsies. Differences between non-inflamed (PDAI histological subscore 0-1, dark grey, n=22) and inflamed (PDAI histological subscore ≥2, light grey, n=6) biopsies. A: Representative dot plot of α4β7 gating on CD3⁺ cells based on fluorescence minus-one (FMO) control. B: Relative expression of α4β7 on CD3⁺ T-cells, CD4⁺ T-cells and CD8⁺ T-cells to mean % per panel. C: Distribution of memory (CD45RO⁺), naïve (CD45RO⁻CCR7⁺) and regulatory (CD127^{low}CD25^{high}) phenotypes within CD4⁺ α4β7⁺ T-cells of non-inflamed (n=11) and inflamed (n=2) biopsies. D: Percentage of CD3⁺CD8⁺ T-cells expressing CD103 within non-inflamed and inflamed biopsies. E: Relative expression of α4β7 on CD3⁺ T-cells, CD4⁺ T-cells and CD8⁺ T-cells to mean % per panel between patients without any episode of pouchitis (dark grey, n=5), patients with chronic pouchitis without active inflammation (white, n=12) and patients with active pouchitis (light grey, n=6). Statistical testing was performed using Mann-Whitney U test or Kruskal-Wallis with Dunn's correction for multiple testing. Data presented as median. A p-value <0.05 was considered statistically significant (*p<0.05, **p<0.01, ****p<0.0001). PDAI, Pouch Disease Activity Index.

differences between jejunum (high levels), ileum (intermediate levels) and colon (low levels).[27-29] In the pouch mucosa, 54% (IQR 46-66%) of α4β7 expressing T-cells co-expressed CCR9. Abundance of these gut-homing α4β7⁺CCR9⁺ T-cells was significantly higher in the pouch mucosa compared to the peripheral blood, both in patients with and without inflammation (Fig. 4A, B). In line with the overall α4β7⁺ cells, the relative expression of α4β7 and CCR9 on CD3⁺ T-cells was lower in inflamed compared with non-inflamed pouches, albeit not statistically significant (Fig. 4C). Gene expression of

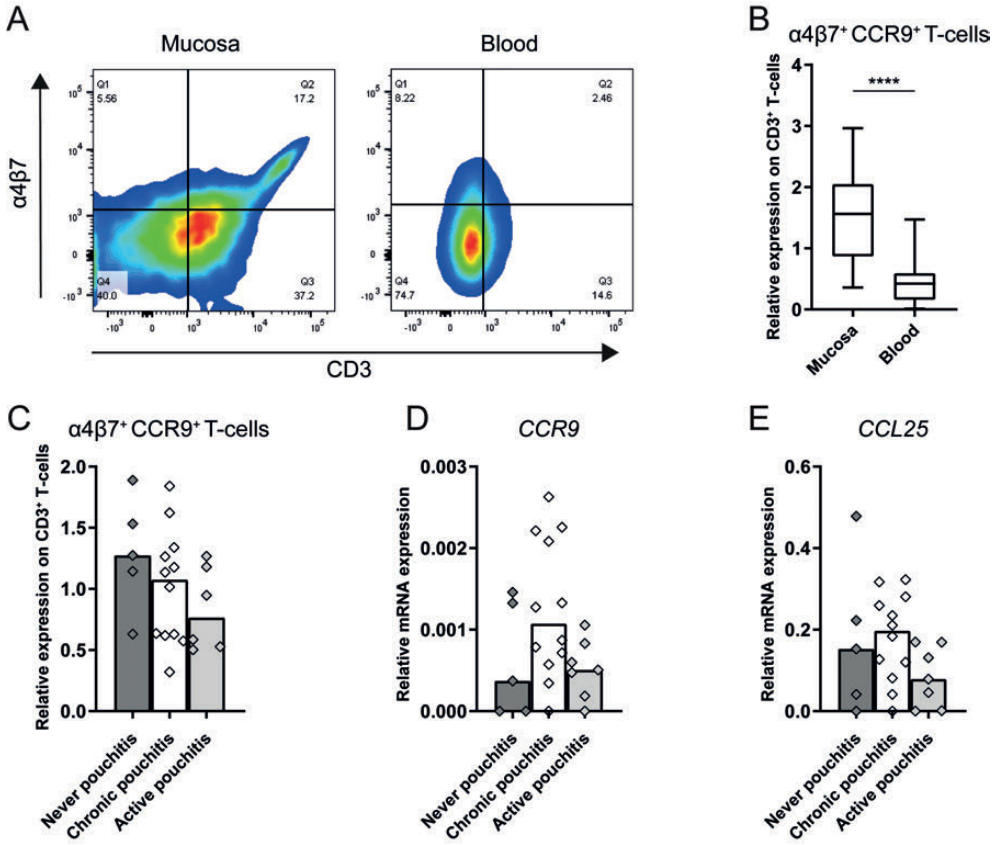

Figure 4. T-cells co-expressing integrin α4β7 and CCR9 are more abundant in pouch mucosa than peripheral blood. Flow cytometry of pouch biopsies. A: Representative dot plot of α4β7 and CCR9 expression on CD3+ T-cells in mucosa (left) and peripheral blood (right). B: Difference between CCR9+α4β7+ expression on peripheral blood and tissue-infiltrating CD3+ T-cells (n=28). C-E: Comparison of no pouchitis (dark grey, n=5), chronic pouchitis without active inflammation (white, n=12) and pouchitis with active inflammation (light grey, n=6 (C), n=7 (D, E)). C: Relative expression of α4β7 and CCR9 on CD3+ T-cells to mean % per panel. D: Relative mRNA expression of *CCR9* in pouch mucosa. E: Relative mRNA expression of *CCL25* in pouch mucosa.

CCR9 did not differ significantly between groups but seemed increased upon chronic inflammation (Fig. 4D).

Chemokine CCL25 is produced by the small intestinal epithelium, where it attracts cells expressing its ligand CCR9. Its expression is increased upon inflammation in IBD, both in the small intestine as well as in colonic tissue.[27-29] We addressed whether mRNA expression of *CCL25* was altered upon inflammation and found that this was not the case (Fig. 4E).

The majority of circulating T-cells expressing integrin α4β7 display a naive phenotype

Local inflammation in the small intestine can lead to recirculation of lymphocytes in the peripheral blood.[29] To investigate whether also systemic changes in lymphocyte subsets occur during pouch inflammation, peripheral blood was sampled before endoscopy. There was no increase in total circulating lymphocyte counts between patients with and without inflammation in the pouch (Supplementary Fig. 4B). In line with a previous report, integrin α4β7 was present on significantly more $CD8^+$ T-cells compared with $CD4^+$ T-cells in peripheral blood ($p<0.001$; Fig. 5A).[30] There was a similar decreasing trend in α4β7 expressing T-cells in peripheral blood upon inflammation as seen in the mucosa (Fig. 5B). This was consistent when comparing patients with and without pouchitis based on the cut off of the combined PDAI≥7, including histological, endoscopic and clinical findings (Supplementary Fig. 4).The majority of α4β7 expressing T-cells in blood were naive T-cells, which did not differ between patients with and without pouchitis (Fig. 5C). Overall, we did not confirm a recirculation of $\alpha4\beta7^+$ T-cells in the peripheral blood in case of inflammation in the pouch mucosa.

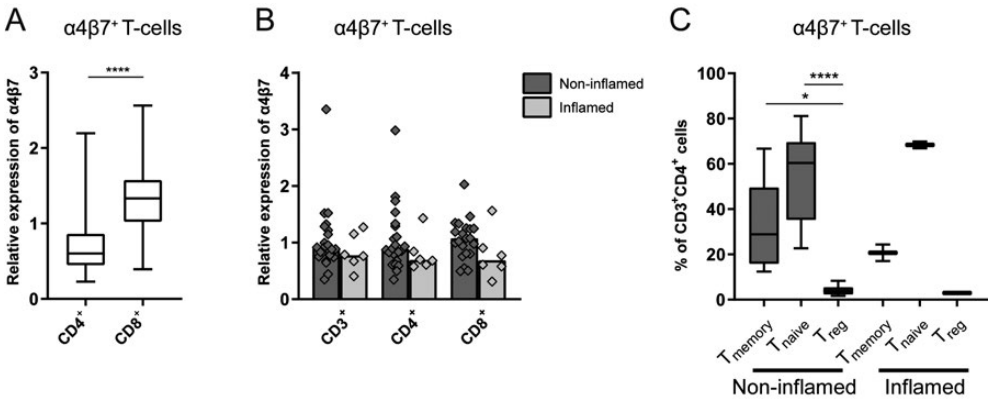

Figure 5. No differences in α4β7-expressing T-cells in blood. Flow cytometry of peripheral blood samples. A: Difference in expression of integrin α4β7 between $CD4^+$ and $CD8^+$ cells. Relative expression to mean % per panel (n=28). B: Relative expression of α4β7 on $CD3^+$, $CD4^+$ and $CD8^+$ T-cells to mean % per panel. Differences between patients with non-inflamed (PDAI histological subscore 0-1, dark grey; n=22) and inflamed (PDAI histological subscore ≥2, light grey; n=6) biopsies. C: Distribution of memory ($CD45RO^+$), naïve ($CD45RO^-CCR7^+$) and regulatory ($CD127^{low}CD25^{high}$) phenotypes within $CD4^+$ $\alpha4\beta7^+$ T-cells of non-inflamed (n=11) and inflamed (n=2) biopsies. Statistical testing was performed using Mann-Whitney U test or Kruskal-Wallis with Dunn's correction for multiple testing. Data presented as median. A p-value <0.05 was considered statistically significant (*$p<0.05$, ****$p<0.0001$).

Discussion

Despite the high incidence of pouchitis occurring in UC patients after bowel surgery, understanding of the underlying immunological processes and subsequently of treatment targets is still lacking. In this study, we show that gut-homing T-cells expressing α4β7 are abundant in pouch mucosa and expression of MAdCAM-1 is increased upon inflammation in the pouch. Conversely, instead of a concordant increase in the proportion of integrin α4β7 expressing T-cells in inflamed compared to non-inflamed mucosa, we rather observed a trend in decreased frequencies of these cells.

Several studies have shown that a substantial number of lamina propria T-cells do express α4β7 as well as CCR9. Moreover, this expression is increased in IBD compared with healthy controls though data on changes as a result of active inflammation in human ileum are scarce.[23,28,31,32] The observed increase in MAdCAM-1 expression in inflamed pouch mucosa in our samples would suggest an increased influx of infiltrating cells expressing its ligand α4β7, but our data do not support this. There are several putative explanations for this rather counter-intuitive finding. First, Meenan *et al.* showed decreased proportions of α4β7 in Crohn's disease (CD) and UC mucosa compared with healthy controls,[23] and Papadakis *et al* showed a decrease in CCR9 expression in inflamed mucosa compared with adjacent uninflamed mucosa.[29] Possibly, expression of homing markers is downregulated after homing into the assigned tissue. Second, integrin α4β7 is expressed on a variety of immune cells including T-cells, B-cells, eosinophils, natural killer (NK) cells, and monocytes, which could be another explanation for the discordant MAdCAM-1 and observed α4β7 expression on T-cells in the mucosa.[33] In the present study we focused on lymphocytes infiltrating the intestine. However, innate immune cells expressing α4β7 may also play a role. For example, a recent study by Schleier *et al.* proposed a role for non-classical monocyte homing to the gut via integrin α4β7.[34] Of note, as we did not correct for total endothelial cells, it is unclear whether the higher expression of MAdCAM-1 is a result of increased expression by the endothelial cells itself or rather an increase in vascularization. The interference of antibodies against both integrin α4β7 and β7 is a drawback of this study, which makes it impossible to interpret the percentages of α4β7-positive cells when comparing all samples. However, current analysis did give an indication of the proportions of α4β7-positive cells between inflamed and non-inflamed tissue.

The definition of pouchitis using the PDAI is based on a combination of clinical, endoscopic and histological parameters. However, especially in case of chronic pouchitis, patients can be classified as having pouchitis based on clinical symptoms while having only mild histological signs of inflammation. Because we aimed to delineate lymphocyte subsets in active inflammation, we based our distinction of inflammation on histology. Pouchitis was relatively mild in most cases, with severe neutrophilic granulocyte infiltration or severe ulcerations occurring in none of the patients. This could explain the relatively small differences we observed between inflamed and non-inflamed tissue. Additionally, the small sample sizes could have led to a lack of power to show differences.

Several case reports and one retrospective cohort have shown positive endoscopic responses on treatment with vedolizumab in patients with chronic refractory pouchitis.[16,35] The lack of increased accumulation of α4β7-expressing T-cells in inflamed pouches may seem counter-intuitive for the use of integrin-blocking therapy. However, it has been suggested that the efficacy of vedolizumab might differ based on the composition of cells expressing α4β7 in the inflamed tissue, both regarding T-helper subsets, as well as other cell types.[36] It was reported previously that lower initial frequencies of α4β7-expressing T-cells were associated with a better clinical response on vedolizumab treatment.[37] Again, this may be related to downregulation or the presence of other cell types expressing α4β7. Treatment with anti-integrin therapy or anti-MAdCAM1 antibodies could therefore still be favourable. The ongoing EARNEST phase 4 study investigating the clinical benefit of vedolizumab in patients with pouchitis may shed more light on this (ClinicalTrials.gov Identifier NCT02790138).

Taken together, our findings indicate that gut-homing trafficking mechanisms are operational in pouchitis, but the classic hypothesis that the chronic inflammatory state is maintained by an increased accumulation of α4β7-expressing effector T-cells is not supported by our data. Longitudinal studies specifically addressing α4β7 expression on several infiltrating cell types and changes thereof in patients treated with specific (α4)β7 or MAdCAM-1 blockers are needed to further elucidate the role of gut-homing in pouchitis.

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Supplementary Tables

Supplementary Table 1. Pouchitis Disease Activity Index (PDAI) of included patients

	Inflamed (n=7)	**Non-inflamed (n=22)**
Clinical [median (IQR)]	**2 (1-4)**	**3 (1-4)**
Stool frequency [n (%)]		
Usual postoperative stool frequency	1 (14)	4 (18)
1-2 stool/day > postoperative usual	2 (29)	10 (46)
≥3 stools/day > postoperative usual	4 (57)	8 (36)
Rectal bleeding [n (%)]	1 (14)	10 (46)
Faecal urgency or abdominal cramps [n (%)]		
None	4 (57)	12 (55)
Occasional	2 (29)	6 (27)
Usual	1 (14)	4 (18)
Fever (temperature > 37.8°C) [n (%)]	1 (14)	0 (0)
Endoscopic inflammation [median (IQR)]	**5 (4-6)**	**1 (0-3)**
Oedema [n (%)]	6 (86)	7 (32)
Granularity [n (%)]	5 (71)	7 (32)
Friability [n (%)]	6 (86)	8 (36)
Loss of vascular pattern [n (%)]	6 (86)	7 (32)
Mucus excudate [n (%)]	5 (71)	5 (23)
Ulcerations [n (%)]	5 (71)	8 (36)
Acute histological inflammation [median (IQR)]	**2 (2-3)**	**1 (0-1)**
Polymorphonuclear leucocyte infiltration [n (%)]		
None	0 (0)	9 (41)
Mild	0 (0)	13 (59)
Moderate + crypt abcesses	7 (100)	0 (0)
Severe + crypt abcesses	0 (0)	0 (0)
Ulcerations per low power field [n (%)]		
0%	5 (71)	22 (100)
0-25%	2 (29)	0 (0)
25-50%	0 (0)	0 (0)
>50%	0 (0)	0 (0)
Total PDAI score [median (IQR)]	**11 (7-12)**	**5 (3-6)**

Supplementary Table 2. Used antibodies for flow cytometry
Panel 1

Antibody	Fluorochrome	Clone	Company	Dilution
CD45RO	APC-H7	UCHL1	Pharmingen	1:30
CD3	AF700	UCHT1	Biolegend	1:200
CD4	PerCP	L200	Becton Dickinson	1:20
CD8	BV500	RPA-T8	Becton Dickinson	1:20
α4β7	DyLight 650	n.a.	*	1:500
β7	BV421	FIB504	Becton Dickinson	1:500
CD103	BV605	Ber-ACT8	Biolegend	1:400
CCR9 (CD199)	FITC	112509	R&D systems	1:10
CCR10	PE	314305	R&D systems	1:50
CCR6 (CD196)	PE-Cy7	11A9	Becton Dickinson	1:20

Panel 2

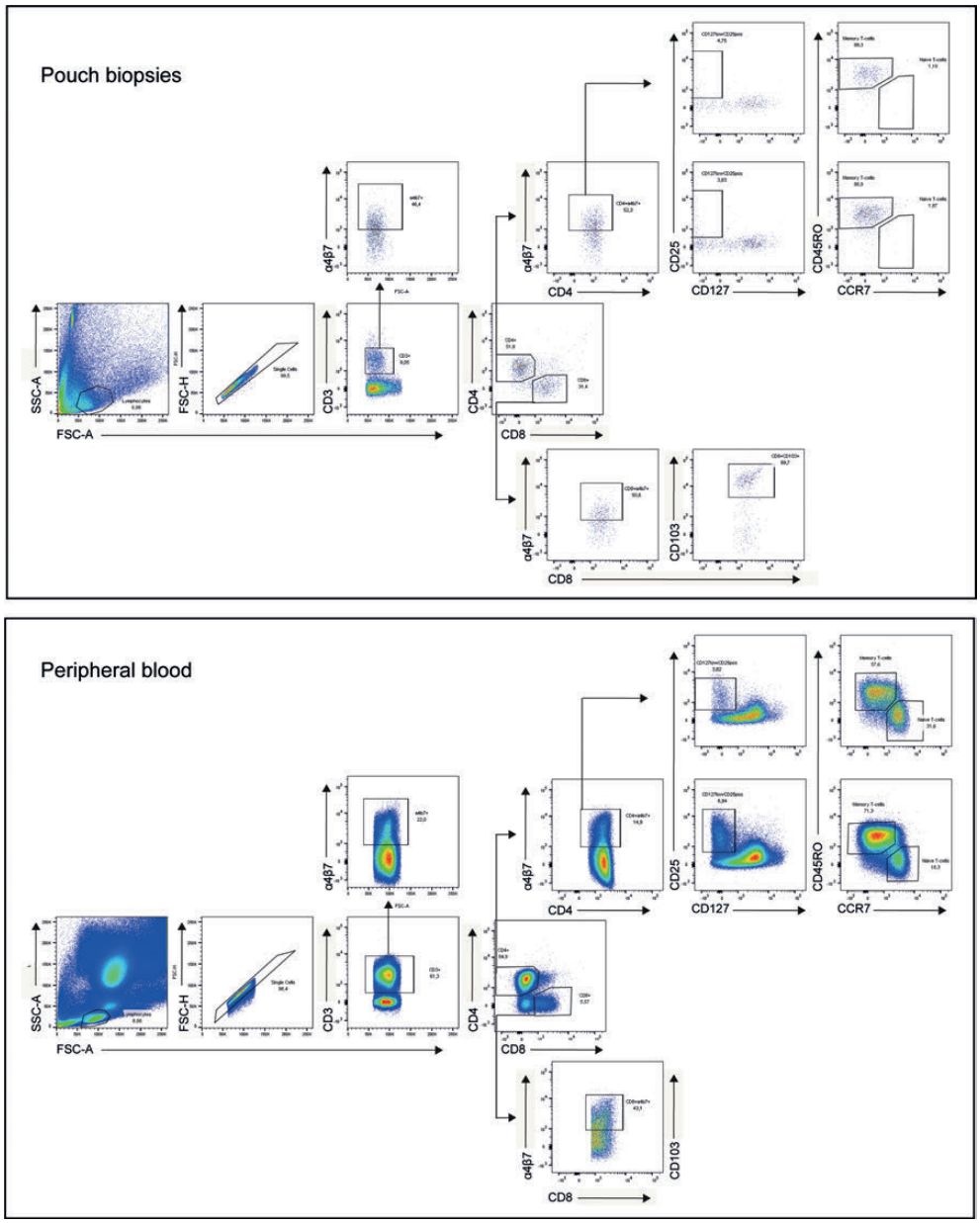
Antibody	Fluorochrome	Clone	Company	Dilution
Live/dead	**	n.a.	ThermoFisher Scientific	1:1000
CD45RO	APC-H7	UCHL1	Pharmingen	1:30
CD3	AF700	UCHT1	Biolegend	1:200
CD4	PerCP	L200	Becton Dickinson	1:20
CD8	BV500	RPA-T8	Becton Dickinson	1:20
α4β7	DyLight 650	n.a.	*	1:500
CD103	BV605	Ber-ACT8	Biolegend	1:400
CCR9 (CD199)	FITC	112509	R&D systems	1:10
CD25	PE	BC96	Biolegend	1:25
CD127	BV421	A019D5	Biolegend	1:50
CCR7	PE-Cy7	G043H7	Biolegend	1:50

* For staining of integrin α4β7, monocolonal antibody vedolizumab (Takeda, Tokyo, Japan) was directly labeled with a fluorochrome using the DyLight 650 Microscale Antibody Labeling Kit, according to manufacturer's protocol (Thermo Scientific/Life Sciences).
** Excitation maximum ~595 nm, emission of ~615 nm

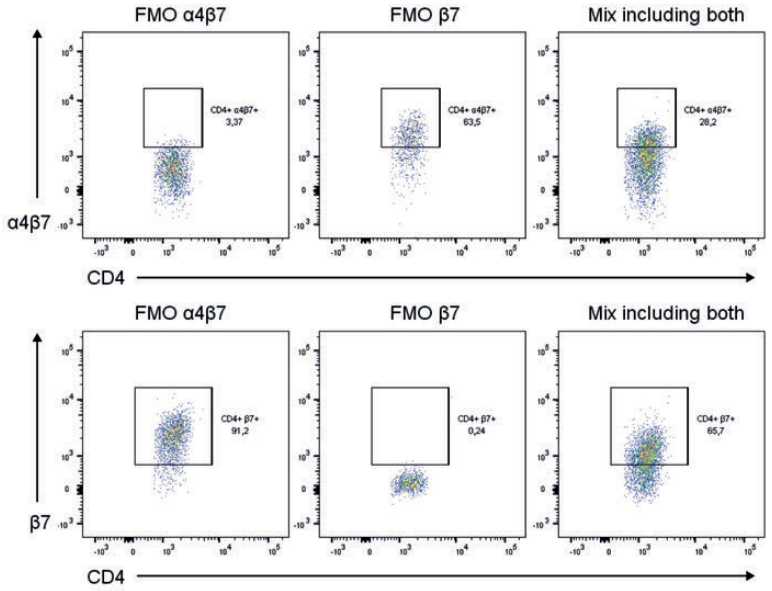
Supplementary Table 3. Primer sequences

Gene	Forward sequence	Reverse sequence
MAdCAM-1	QT00017367 (Qiagen)	
CCL25	CAAAGCTCCACCACAACACG	ATGGAGCCCAGAAATGAGCC
CCL28	AATGCAGCAGAGAGGACTCG	TGACAGCAGCCAAGTCACAA
CCR9	AGAGCAGGCTTGCATCTGAC	GCCATAGTCATCAGCCATGTT
CCR10	GAGGCCACAGAGCAGGTTTC	CATCGGCCTTGTAGCAAAGC
36B4	TCATCAACGGGTACAAACGA	GCCTTGACCTTTTCAGCAAG
GAPDH	GTCAGTGGTGGACCTGACCT	TGAGCTTGACAAAGTGGTCG

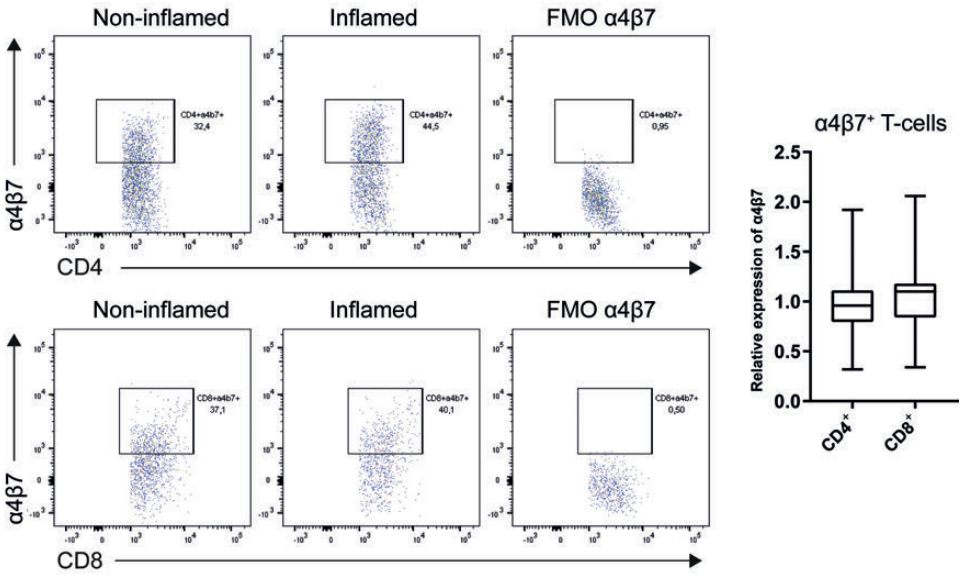
Supplementary Figures



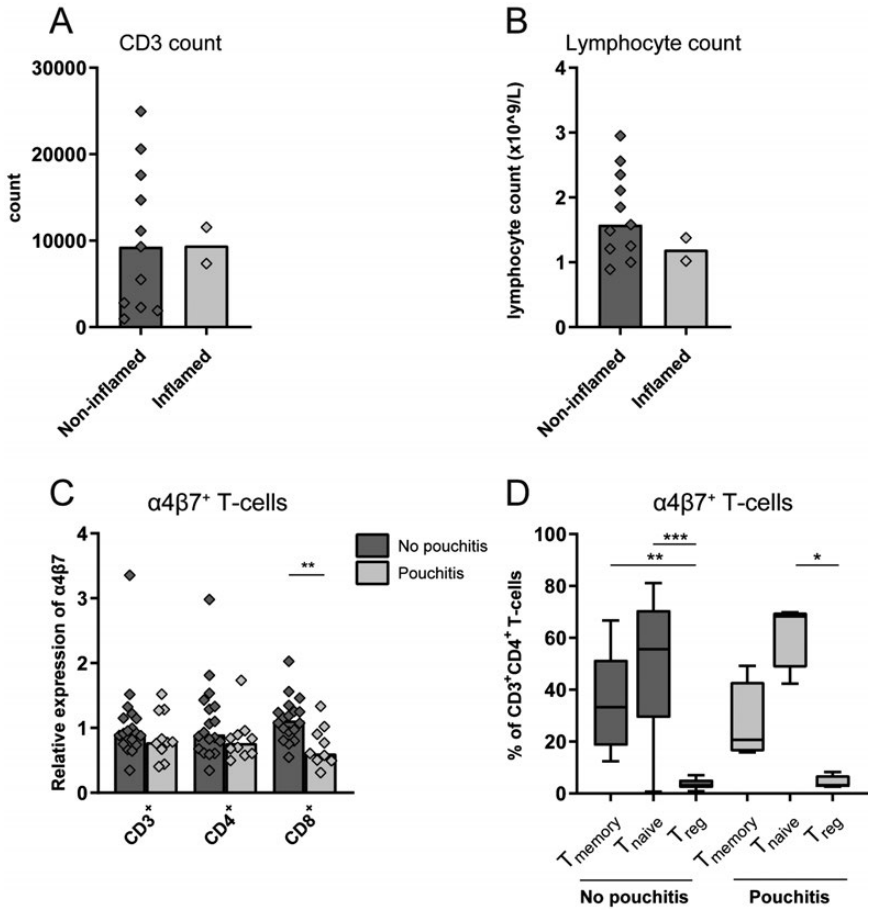
Supplementary Figures 1. Gating strategy of pouch biopsies and peripheral blood samples.



Supplementary Figure 2. Flow cytometry on colon derived mononuclear cells, gated on $CD3^+$ $CD4^+$ cells. Percentage of α4β7 positive cells (upper panel) and β7 positive cells (lower panel) in fluorescence minus one (FMO) control for α4β7 (left), FMO for β7 (middle) and mix including both antibodies (right).



Supplementary Figure 3. Flow cytometry on pouch biopsies. Representative dot plots of α4β7 positive cells on $CD4^+$ and $CD8^+$ T-cells in inflamed (left) and non-inflamed (middle) biopsies, gated based on fluorescence minus one (FMO) control. Quantification of relative expression of α4β7 on $CD4^+$ T-cells and $CD8^+$ T-cells to mean % per panel (n=28).



Supplementary Figure 4. A: Absolute cell counts of $CD3^+$ cells in non-inflamed (n=11) and inflamed (n=2) pouch biopsies. B: Absolute lymphocyte counts in peripheral blood samples of patients with non-inflamed (n=11) and inflamed (n=2) pouch biopsies. C: Relative expression of α4β7 on $CD3^+$, $CD4^+$ and $CD8^+$ T-cells in patients without pouchitis (PDAI<7, n=22, dark grey), or with pouchitis (PDAI ≥7, n=9, light grey). D: Distribution of memory ($CD45RO^+$), naïve ($CD45RO^-$ $CCR7^+$) and regulatory ($CD127^{low}CD25^{high}$) phenotypes within $CD4^+$ $\alpha4\beta7^+$ T-cells of non-inflamed (n=11) and inflamed (n=2) biopsies. Statistical testing was performed using Mann-Whitney U test or Kruskal-Wallis with Dunn's correction for multiple testing. Data presented as median. A p-value <0.05 was considered statistically significant (*p<0.05, **p<0.01, ***p<0.001).