Mucosal T cells in IBD

FROM IMMIGRANTS TO RESIDENTS

Britt Roosenboom

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From immigrants to residents

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Promotor

PROF. DR. J.P.H. DRENTH

Copromotoren

DR. C.S. HORJUS TALABUR HORJE (RIJNSTATE)

DR. P.J. WAHAB (RIJNSTATE)

DR. E.G. VAN LOCHEM (RIJNSTATE)

Manuscriptcommissie

PROF. DR. I.D. NAGTEGAAL

PROF. DR. I.J.M. DE VRIES

PROF. DR. R.K. WEERSMA (UNIVERSITAIR MEDISCH CENTRUM GRONINGEN)

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CHAPTER 1

General introduction

The intestinal mucosa is an immunologically dynamic environment in which the gut constantly interacts with commensal microorganisms, dietary antigens and pathogens of different nature. Despite this excessive exposure to foreign antigens, the gut mucosa maintains intestinal homeostasis by utilizing the mucosal immune system. This comprises an ingenious immune network including gut-associated lymphoid tissue, resident immune cells, cytokines, chemokines, antimicrobial peptides and commensal bacteria. Disruption of this finetuned gut mucosal immune network, leads to chronic inflammation also known as inflammatory bowel disease (IBD).

Clinical aspects of inflammatory bowel disease

IBD is an overarching term for different clinical phenotypes defined according to the Montreal classification consisting of: Crohn´s disease (CD), ulcerative colitis (UC), IBD unclassified and IBD indeterminate.[1,2] When considering such broad terms, it is important to note that there is not just one CD or one UC entity, but rather numerous variations that differ in clinical presentation and behaviour, which likely reflects differences in underlying pathogenic mechanisms.[3,4] Therefore clinical classification should be rethinked in this new era of personalized and precision medicine.[5] However until now definition and classification of the different IBD phenotypes is made according to the affected area in the gastrointestinal tract, the depth of inflammation in the intestinal wall and histological findings. Any part of the gastrointestinal tract can be affected in CD, but most often disease activity is seen in the terminal ileum and colon. Inflammation is distributed in a patchy-pattern (inflammation is separated by uninvolved skip areas) and penetrates through the entire bowel wall which can lead to fistulas and abscesses. UC typically affects the most inner layer of the colonic wall and is most often continuous extended from rectum to proximal segments of the colon. Histopathologic analyses of mucosal biopsies showed a wide overlap between CD and UC. The histological diagnosis of CD is largely based on the finding of granulomas in addition to the presence of cytological and architectural changes. Segmental distribution of crypts or crypt atrophy, segmental distribution of mucin depletion, mucin preservation at the edge of an ulcer or crypts surrounding neutrophils, the occurrence of focal inflammation simultaneously with severe patchy inflammation have a significant discriminative value in favor of CD.[6,7]

Patients with IBD suffer from a chronic disease characterized by unpredictable episodes of relapse and remission. Diarrhea, blood in their stool, abdominal pain, urgency, weight loss, fatigue and fever are frequent reported complaints. Next to these symptoms, IBD patients can suffer from long term complications caused by disease activity, such as development of strictures and obstruction with need for surgery and hospitalization. Over the last twenty years there have been substantial improvements

in treatment options available, providing effective and sustained remission for many patients whilst appearing to delay or prevent progression to surgery.[8,9,10] Despite the increase in therapeutic options, significant challenges remain, including maintaining therapeutic response on the longterm, positioning the right drug for the right patient and avoiding toxicity.

Immune therapy related colitis

Immune checkpoint inhibitors are recently introduced antibodies that target specific downregulators of the anti-cancer immune response: cytotoxic T-lymphocyte antigen-4 (CTLA-4), programmed cell death protein-1 (PD-1) and its ligand (PD-L1). CTLA-4 inhibitors (ipilimumab, tremelimumab) and PD-1/PD-L1 inhibitors (nivolumab, pembrolizumab) are associated with immune-related adverse events due to over-activation of the immune system and affect most commonly the gastrointestinal tract. This leads to another type of inflammation in the gut, the so called immune therapy related colitis,[11,12] which is frequently reported and displays endoscopic and histological similarities when compared to Crohn's disease and ulcerative colitis. Anti-CTLA-4 agents induced enterocolitis with a more acute profile without features of chronicity (basal plasmacytosis, atrophy, distortion and branching of crypts), whereas anti-PD-1/PD-L1 inhibitors can lead to enterocolitis with an acute and/or chronic pattern.[13,14]

Pathogenesis of inflammatory bowel disease

The pathophysiology of IBD involves complex epithelial, genetic, microbial, environmental and immune factors.[15,16] First, early studies suggested that the mucus layer was denuded in Crohn's disease[17] caused by a reduction in secretory cells and downregulation of a colonic goblet-cell-secreted protein, demonstrated by single cell RNA sequencing. This downregulation lead to abnormalities in mucus layer formation, increased colonization and invasion of microbiota, and breakdown of the epithelial barrier in active UC.[18] Stromal cells (nonhematopoietic mesenchymal cells) which play an important role in fibrosis and wound healing were also suggested to be of value in enhancing epithelial barrier function in IBD. Next to these epithelial factors, genomewide association studies have identified more than 240 risk variants that affect intracellular pathways recognizing microbial products (e.g. NOD2), the autophagy pathway which facilitates recycling of intracellular organelles and removal of intracellular microorganisms (e.g. ATG16L1), genes regulating epithelial barrier function (e.g. ECM1) and pathways regulating innate and adaptive immunity (e.g. IL23R and IL10).[19] Although many individuals carry IBD-associated risk loci, only a small population develops IBD. Therefore additional

alterations in the gut microbiota (reduction in the total number, diversity and richness of microbial species), environmental factors (e.g. diet, smoking, psychological stress) and changes in the innate and adaptive immune system are supposed to be required for the development of IBD.

Mucosal immunity in IBD

The immune system can be compartmentalized into the innate and adaptive part. Innate immune cells detect microbial products or patterns and include granulocytes, macrophages and dendritic cells. They regulate tissue homeostasis, repair, remodeling and microbial defense. The adaptive immune cells include B- and T cells that recognize specific antigens.

T lymphocytes: immigrants and residents

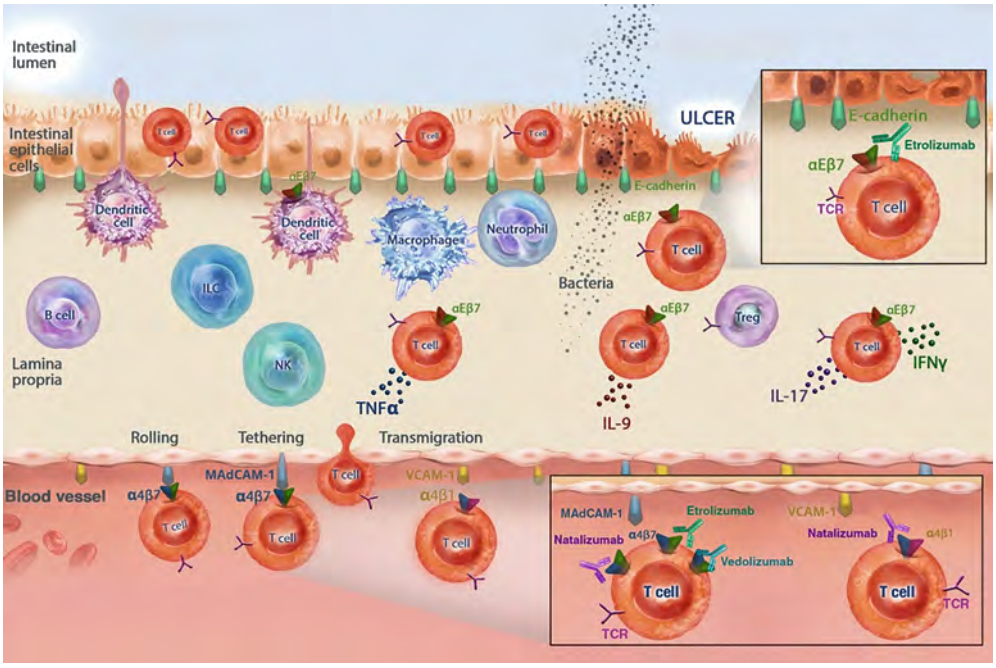
Trafficking of T lymphocytes to the gut is a tightly regulated process important for maintaining homeostasis and initiating immune responses. After leaving the thymus, naïve T lymphocytes, expressing L-selectin (CD62L), CCR7 and low levels of α4β7 integrin, face their cognate antigen presented by dendritic cells in the gut-associated lymphoid tissue (GALT), smaller lymph aggregates and mesenteric lymph nodes. This drives activation, proliferation and imprinting of T lymphocytes with a gut homing phenotype. Some T lymphocytes develop into central memory T cells expressing L-selectin (CD62L) and chemokine receptor 7 (CCR7), whereas others become effector T lymphocytes expressing α4β7 and CCR9 or regulatory T lymphocytes. The central memory and effector T lymphocytes leave to GALT to enter the lamina propria via the systemic circulation and produce inflammatory cytokines providing protection from microbial infection.[20]

Migration of T cells via high endothelial venules (HEVs) in gut-associated lymphoid tissue (GALT) and post-capillary venules in the lamina propria, to the gut mucosa begins with loose tethering to and subsequent rolling along the endothelial wall. This process slows T lymphocytes to allow the cells to sense chemokines presented by the endothelium. Chemokines induce T cell activation (integrin activation) leading to lymphocyte arresting at the endothelium through interaction of integrins with their addressins. Interaction between α4β7 and CD62L with (carbohydrate-modified) MAdCAM-1, α4β7 and α4β1 (particularly in the inflamed ileum) with VCAM-1, and LFA-1 with ICAM-1 lead to a firm arrest of these T lymphocytes to endothelial cells, after which lymphocytes undergo morphological changes and are able to extravasate to secondary lymphoid tissue or the intestinal lamina propria.[21] Regulatory T lymphocytes which mediated a suppressive function can be generated in gut-draining lymph nodes and proliferate after homing to the intestine.

After homing to lymphoid aggregates, T lymphocytes migrate to the lamina propria. Subsequently, T lymphocytes downregulate integrin α4 expression and upregulate integrin αE (CD103) expression driven by transforming growth factor beta (TGFb). αE together with β7 integrin, can adhere to E-cadherin expressed on basolateral epithelial surface. This change in homing markers lead to long-term residency in the epithelium. The majority of $\alpha E\beta7^+$ T lymphocytes in the intestine are $CD3^+CD8^+$ T lymphocytes and neither B lymphocytes, nor macrophages express the αEβ7 integrin. αEβ7 is expressed on 90% of intraepithelial lymphocytes (IELs) and 40% of T lymphocytes in the lamina propria.[22,23]

In IBD a dysregulated T lymphocyte response is often implicated as key mediator of chronic inflammation (see **Figure 1**), explained by the growing number of successful therapeutic approaches targeting these cells in IBD. Next to high numbers of effector memory T lymphocytes, increased percentages of $CD4^+$, naïve, central memory and regulatory T lymphocytes and decreased percentages of $CD8^+$ and $CD103^+$ T lymphocytes were seen in biopsy specimens of newly diagnosed IBD patients.[24,25] Within the mucosal αE^+ T-lymphocyte population, a proinflammatory role was allocated exclusively for the $CD4^+$ subset, whereas data are conflicting regarding to the profile of

Figure 1 *Overview of the role of integrins and immune cells in the pathogenesis of IBD.*[29]



αE^{+}CD8^{+} T lymphocytes, with studies both suggesting proinflammatory and regulatory functions.[22,23,26,27,28] Different subsets of T lymphocytes (and their products, cytokines and chemokines) in different IBD patients serve as therapeutic targets and could potentially guide the use of drugs.

Therapeutic targets in inflammatory bowel disease

While the pathophysiologic mechanisms contributing to development of IBD have not been fully clarified, current research suggests that genetically-susceptible individuals respond to environmental factors with alterations in the innate and adaptive immune responses at the gastrointestinal barrier. These findings provided the basis for development of new therapies and led to an expansion of the former therapeutic arsenal consisted of 5-aminosalicyclic acid, corticosteroids, thiopurines and methotrexate.[30] The first patient with severe Crohn's disease not responding to conventional therapy, received anti-tumor necrosis factor (TNF) antibodies (Infliximab) at the Academic Medical Centre (AMC) in Amsterdam, and had a spectacular clinical response.[31] This was the revolutionary beginning of the treat-to-target therapeutic era in IBD. Shortly after, other anti-TNF antibodies including Adalimumab, Golimumab or Certolizumab entered the stage. Also antibodies antagonizing actions of other proinflammatory cytokines, such as IL-12 and IL-23 with a common p40 subunit (Ustekinumab) proved their effectiveness.[32] Briakinumab, Brazikumab, Risankizumab, Mirikizumab and Guselkumab are also antibodies blocking interleukin-12 (IL-12) and interleukin-23 (IL-23).

Next to inhibiting pro-inflammatory cytokines, anti-integrin antibodies are effective in reducing inflammation in IBD.[33] Anti-integrin antibodies block the actions of integrins on circulating immune cells and those of their receptors on endothelial cells. Vedolizumab is approved as an antibody that targets α4β7-integrin expressed on T lymphocytes thereby blocking binding to mucosal addressin cell adhesion molecule-1 (MAdCAM-1) on endothelial cells in the gastrointestinal tract. Abrilumab is also an α4β7-integrin inhibitor which can be administered subcutaneously.

Inhibition of the β7-subunit with Etrolizumab is currently under investigation in numerous studies, however since negative results were reported, the study program for UC patients was interrupted. One important aspect of Etrolizumab is the fact that it targets not only α4β7-integrin, but also αEβ7-integrin mediated interaction between intraepithelial lymphocytes and E-cadherin expressed by enterocytes, thereby blocking retention of T lymphocytes which already populate in the inflamed intestinal mucosa.[34] Blockade of αEβ7 integrin affect both proinflammatory T lymphocytes as regulatory T lymphocytes. This could potentially explain the lack of desired effect compared to Vedolizumab. Besides integrin inhibition, MAdCAM-1 expressed on the endothelium of venules in intestinal lamina propria of the gut and gut-associated tissue, can be blocked by

Ontamalimab. Trials investigating efficacy of Ontamalimab were interrupted by Takeda due to commercial issues, rather than drug efficacy or safety.[35]

Next to monoclonal antibodies, small molecules are under investigation for IBD therapy. They allow a more convenient oral formulation while providing specific molecular targeting. The armamentarium is further enhanced by the approval of JAK inhibitors, which block intracellular signaling by inhibiting tyrosine kinases. Tofacitinib, an inhibitor of JAK1 and JAK3, is accepted for the treatment of UC patients.[36] Besides all afore mentioned therapeutic options, sphingosine-1-phosphate receptors modulators, phosphodiesterase inhibitors and oligonucleotide-based therapeutics are under investigation for the treatment of IBD patients. These novel therapeutics will further enhance the scope of currently available targeted drugs for the treatment of IBD.[30]

Not all invented immunological targets has been turned out to be suitable as treatment-target in IBD. Integrins have the potential to be gut-selective, but they also are involved in leucocyte migration into other tissue than the gut, for example the MoAb Natalizumab, blocking α4 (both α4β7 and α4β1), leads to autoimmune encephalitis. In addition to current therapeutic strategies blocking pro-inflammatory cytokines, lymphocyte gut homing en Janus Kinases, several novel treatments aimed at strengthening the immunoregulatory pathways (e.g. interfering with regulatory T cells, TGFβ, IL-10, GM-CSF and mesenchymal stem cells) are proposed for the treatment of IBD, further enhancing the immense and thereby troublesome target-driven therapeutic arsenal for the clinician.

Since patients with similar clinical phenotypes can have different activated inflammatory pathways, they respond different to the same therapy. Furthermore, changes of activated inflammatory pathways have been observed during disease course, where helper T lymphocytes can acquire regulatory functions upon chronic stimulation in inflamed tissue (plasticity of T-lymphocyte subsets). Both phenomena can contribute to the limited efficacy of therapies targeting the immune response and can underline the need for development of immunological biomarkers predicting response to therapy.[37] The scope for these response predictive biomarkers depends on finding the right balance between cutting-edge technology and easy-to-implement clinical tools.[38]

Aim of this thesis

Since not all patients respond to the above mentioned drugs, it is conceivable that personal immunological phenotyping in active inflamed gut mucosa must guide the choice for a specific treatment, in the future. Therefore we investigated the presence of different (potential) immunological treatment targets, including α4β7, αEβ7 and PD-1 on T lymphocytes, next to MAdCAM-1 and PNAd on endothelial cells in UC and CD patients and their relation to disease activity (1). Furthermore we investigated the functional

profile of $CD8^+$ Trm cells since studies suggested both proinflammatory and regulatory functions (2). To complete this thesis, we studied the response predictive value of MAdCAM-1 and PNAd on endothelial cells and $\alpha4\beta7/\alpha E\beta7$ on T lymphocytes in UC patients treated with Vedolizumab (3), as introduction of predictive mucosal biomarkers into clinical practice will enable more precise and individualized strategies for selecting therapeutics in IBD.

Outline

Chapter 1 introduce the background information on T-lymphocyte trafficking to the gut and the framing of this thesis. The presence of $PNAd^+$ high endothelial venules (HEVs) and $MAdCAM\text{-}1^+$ venules in the intestinal mucosa of newly diagnosed patients with ulcerative colitis is demonstrated in chapter 2. We investigate the presence of these venules at diagnosis and during follow-up and study their correlation with disease activity. In chapter 3 we hypothesize that different pre-treatment mucosal markers related to the integrin-dependent T-lymphocyte homing could potentially predict response to $\alpha4\beta7$ integrin inhibition. Discovering such predictors may lead to a more patient tailored treatment in the future.

Next we explore percentages of $\alpha E\beta7^+$ T lymphocytes in intestinal biopsies of newly diagnosed untreated IBD patients at baseline and during follow-up and compare them to healthy controls in chapter 4. In chapter 5 we investigate the functional profile of human tissue-resident memory T (Trm) lymphocytes, both of the CD4 and CD8 lineage, by performing mass cytometry, flow cytometry and RNA-sequencing. It is crucial to understand their function since therapeutic strategies targeting gut Trm lymphocytes are under investigation. The potential pathological role of $\alpha E\beta7^+$ T lymphocytes in IBD patients and their value as a biomarker for the use of anti-$\beta7$ treatment is questioned in chapter 6a and 6b.

Furthermore we demonstrate the presence and role of $PD1^+$ mucosal T lymphocytes in colitis of different etiologies such as: IBD, infectious colitis and immunotherapy-related colitis in chapter 7.

This thesis is completed in chapter 8 with a summary of the results and a discussion on future perspectives on mucosal T lymphocytes subsets and potential therapeutic targets in IBD patients.

Table 1 *Summary of research questions and methodology of this thesis*

Chapter	Research question(s)	Study design	Measures
Part I Homing of T cells in ulcerative colitis			
2	• Demonstrating the presence of PNAd and MAdCAM-1 on venules in the gut mucosa of UC patients at diagnosis and during follow-up • What is the correlation between the expression of PNAd and MAdCAM-1 on colonic mucosal venules with histologic disease activity at diagnosis and during disease flares? • Are PNAd and MAdCAM-1 potential predictors of disease course in patients with UC?	Retrospective cohort study	Expression of PNAd and MAdCAM-1 on venules
3	• Are mucosal markers related to integrin dependent T-lymphocyte homing of response predicitve value prior to treatment with Vedolizumab in UC patients?	Prospective cohort study	Expression of α4β7 on T lymphocytes and PNAd and MAdCAM-1 on venules
Part II Resident T-cell population in IBD			
4	• Demonstrating the presence of different intestinal $CD103^+$ T-cell subsets in newly diagnosed, untreated IBD patients and healthy controls at baseline and during follow-up	Prospective cohort study	$CD103^+$ T-cell subpopulations in blood and single cell suspensions from colonic and ileal biopsies
5	• What is the functional profile of human $CD8^+$ Trm cells in healthy control subjects and patients with ileal Crohn's disease?	Cross sectional study	Flow-, imaging mass cytometry and RNA sequencing on lamina propria and intraepithelial $CD103^+CD69^+CD8^+$ T cells
6a and 6b	• Can αE levels in IBD patients function as a biomarker for the use of Etrolizumbab? • What is the pathological role of $\alpha E\beta7^+$ T lymphocytes in IBD patients?	Letter	Expert opinion based on earlier findings
7	• How are $PD\text{-}1^+$ T cells distributed in different types of colitis?	Cross sectional study	PD-1 expression by immunohistochemistry and flow cytometry

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PART I

HOMING OF T CELLS IN ULCERATIVE COLITIS

CHAPTER 2

Development of mucosal PNAd$^+$ and MAdCAM-1$^+$ venules during disease course in ulcerative colitis

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Britt Roosenboom

Ellen G. van Lochem

Jos Meijer

Carolijn Smids

Stefan Nierkens

Eelco C. Brand

Liselot W. van Erp

Larissa G.J.M. Kemperman

Marcel J.M. Groenen

Carmen S. Horjus Talabur Horje

Peter J. Wahab

Abstract

PNAd and MAdCAM-1 addressins on venules are of importance in T-cell homing and potential therapeutic targets in ulcerative colitis (UC). Normally, PNAd$^+$ high endothelial venules (HEVs) are only present in lymphoid organs, whereas small numbers of MAdCAM-1$^+$ venules can be seen in non-lymphoid tissue. We aimed to study their presence in the intestinal mucosa of UC patients at diagnosis and during follow-up, and their correlation with disease activity. Colonic biopsy specimens of 378 UC patients were analyzed by immunohistochemistry for CD3, CD20, ERG, MECA-79 (PNAd) and MECA-376 (MAdCAM-1) and compared to healthy controls (HC). The proportion of PNAd$^+$ HEVs in UC at diagnosis was 4.9% (IQR 2.0%–8.3%), while none were detected in HC. During follow-up, PNAd$^+$ HEVs completely disappeared in remission (n = 93), whereas the proportion in active disease was similar to baseline (n = 285, p = 0.39). The proportion of MAdCAM-1$^+$venules in UC at baseline was 5.8% (IQR 2.6–10.0). During follow-up, the proportion in remission was comparable to diagnosis, but upregulated (7.5% (IQR 4.4–10.9), p = 0.001) in active disease. In conclusion, PNAd$^+$ HEVs appear in UC during active inflammation which could thus serve as a marker for disease activity, whereas MAdCAM-1$^+$venules remain present after inflammation is resolved and increase after subsequent flares, reflecting chronicity and potentially serving as a therapeutic target.

Introduction

Ulcerative colitis (UC) is known to have a heterogenic phenotype reflected by differences in disease location and severity, age of disease onset and response to treatment[1]. There are several therapeutic agents available to reduce symptoms or to prevent progression of disease in patients with UC. However, the response to treatment differs, suggesting that distinct inflammatory mechanisms drive the course of the disease[2-4].

In healthy gut mucosa, naive- (T_n) and central memory T cells (T_{cm}) migrate to secondary lymphoid organs (SLOs) by tethering and rolling on specialized cuboidal formed high endothelial venules (HEVs)[5,6]. This process is facilitated through the binding of L-selectin on the surface of T cells to peripheral node addressin (PNAd) on HEVs[7]. Within SLOs, T cells become activated effector cells (T_{em}) and migrate through blood vessels to their site of action, such as the gut mucosa. The adhesion molecule integrin alpha4beta7 ($\alpha4\beta7$) on T_{em} cells plays a crucial role in controlling this migration process to the intestine by binding to mucosal vascular addressin cell adhesion molecule-1 (MAdCAM-1), a 60-kD glycoprotein, which is expressed on venules in Peyer's patches, mesenteric lymph nodes and on flattened venular endothelial cells in the intestinal lamina propria[8]. MAdCAM-1 contributes to lymphocyte homing by serving as a cell adhesion molecule, not only by binding $\alpha4\beta7^+$, but to a lesser extent also by binding L-selectin$^+$ and/or $\alpha4\beta1^+$ lymphocytes to the luminal surface of venules, and as a vascular addressin for the tethering and rolling of lymphocytes[9].

In contrast to the relative absence of T cells in non-inflamed gut mucosa, they are found in high numbers in the inflamed gut of UC patients reflecting the diffuse chronic inflammatory cell infiltrate[10]. A possible critical step needed to generate this infiltrate, is the morphological and functional change of postcapillary venules into HEVs in non-lymphoid tissue. HEVs are proposed to be absent in the non-lymphoid tissue of healthy gut mucosa. Therefore, their presence might serve as a marker of newly formed tertiary lymphoid organs (TLO), with a quite similar histological appearance to SLOs[11]. These newly developed lymphoid organs might facilitate the homing and reactivation of T cells independent of SLOs in chronic inflamed mucosa[12].

Currently, little is known about the presence of PNAd$^+$ and MAdCAM-1$^+$ venules in the colon of UC patients and their role in the pathogenesis and disease course of UC[13]. During active disease in UC patients, the induction of colonic PNAd$^+$ HEVs was associated with a greater influx of T_n and T_{cm} cells and correlated with the intensity of inflammation based on Ulcerative Colitis Disease Activity Index (UCDAI) scores in a small group of patients[14,15]. In another small cohort of patients, MAdCAM-1$^+$venules were suggested to be upregulated in active UC compared to HC, with no differences in numbers of MAdCAM-1$^+$ venules between patients with active disease and remission[16]. These adhesion molecules and vascular addressins are attractive targets in the treatment of UC since they specifically facilitate the migration of lymphocytes to the gut mucosa,

which plays a vital role in the pathogenesis of UC[17]. Anti-α4β7 integrin (Vedolizumab) is an effective therapy to induce and maintain clinical and endoscopic remission[18]. In addition, the effect of antibodies against MAdCAM-1 (Ontamalimab), which prevent the migration of T cells to the gut by blocking the same 'homing pathway' as anti-α4β7 integrin antibodies, has been studied in a phase II trial with promising results[19]. However, not all patients respond to treatments interfering with the homing of T cells by blocking α4β7 and MAdCAM-1[20–22], probably because of the simultaneous presence of $PNAd^+$ HEVs, serving as an entrance for colitogenic T_n cells[23]. In contrast to anti-MAdCAM-1 therapy, anti-PNAd therapies have not yet been investigated in humans.

In the present study, we evaluated the presence of both PNAd on HEVs and MAdCAM-1 on HEVs and flattened venules in the gut mucosa of UC patients at initial diagnosis and during follow-up endoscopy. We aimed to investigate the correlation between the expression of PNAd and MAdCAM-1 on colonic mucosal venules with histologic disease activity at diagnosis and during disease flares and their potential to predict the course of disease in patients with UC.

PATIENTS AND METHODS

Study Population

Newly diagnosed untreated UC patients between 2000 and 2018 at the Department of Gastroenterology and Hepatology in Rijnstate Hospital in Arnhem, a secondary care center in the Netherlands, were retrospectively included in the study. In patients with symptoms including rectal blood loss, chronic diarrhea, abdominal pain or weight loss, the diagnosis of UC was confirmed by ileocolonoscopy (endoscopic Mayo score ≥1) with colonic biopsies of inflamed mucosa[24].

Endoscopic images and written reports from initial and follow-up ileocolonoscopy were re-assessed for severity of disease using the Mayo score (0–3 scale). We favored the use of a histological score, rather than the endoscopy score, in order to classify the severity of disease because of the high subjectivity of retrospective re-assessment of endoscopic images using the Mayo score. Next to this subjectivity, older endoscopic images are of poorer quality, and thus insufficient for reliable re-assessment. Histopathological assessment of biopsies was performed independently by two reviewers, both blinded for patient characteristics, by using the Geboes score to classify the histologic type and severity of disease, where Grade 0 indicates architectural changes, Grade 1 chronic inflammatory infiltrate, Grade 2 eosinophils (2A) and neutrophils (2B) in the lamina propria, Grade 3 neutrophils in the epithelium, Grade 4 crypt destruction and Grade 5 indicates the presence of erosions and ulcerations in biopsy specimens. Within each Grade (0–5), the stage of severity is then classified in stages arranging from 0 to 3 (no abnormalities to severe changes)[25]. UC patients with a Geboes score ≥ 3.1 (mean-

ing a score higher than stage 1 of Grades 3 or 4 or 5) were identified as patients with histological active disease. Patients were excluded if paraffin-embedded biopsies at baseline and/or follow-up ileocolonoscopy were unavailable for investigation. Follow-up ileocolonoscopy was performed based on routine clinical care, i.e., in case of symptoms suggestive of exacerbation or when surveillance was indicated. The follow-up period was defined as the time between initial diagnosis and the last visit at the outpatient clinic, date of death or loss to follow-up due to migration.

The following variables were extracted from the medical records: patient demographics, disease phenotype (including disease location and behavior) at initial diagnosis according to the Montreal classification[26], the first effective remission-induction therapy following initial presentation, time between initial presentation and first exacerbation (defined by a combination of clinical symptoms, step-up in therapy, biochemical activity and/or confirmed endoscopic disease activity), response to anti-TNF treatment, the need for a subtotal colectomy and medication use at the time of follow-up ileocolonoscopy (i.e. aminosalicylates, topical steroids, systemic steroids, immunomodulators or biologicals).

Healthy controls (HC) who underwent ileocolonoscopy for polyp surveillance or iron deficiency from whom paraffin embedded mucosal biopsies were available were included in the control group. Histopathological analysis confirmed the absence of architectural changes, chronic inflammatory infiltrate, influx of neutrophils and eosinophils in the lamina propria, neutrophils in the epithelium, crypt destruction and erosions or ulcerations in HC.

Immunohistochemistry

3µm-thick sections from formalin-fixed, paraffin-embedded archived blocks of biopsied specimen of colonic mucosa were cut and selected for immunohistochemistry. Besides hematoxylin and eosin (HE, from Klinipath) staining, immunostaining was performed with the following monoclonal antibodies: CD3 (from Novocastra, clone LN10, 1:50, marking T cells), CD20 (from Thermo scientific, clone L-26, 1:125, marking B cells), ERG (from Ventana, clone EPR3864, demonstrating only nuclei of endothelial cells on all blood vessels), MECA-79 (from Santa cruz Biotechnology, clone MECA-79, 1:350, demonstrating 6-sulpho-sialyl Lewis on core-1 branched O-linked sugars (PNAd)), MECA-376 (from Hycult Biotech, clone 314G8, 1:50, marking MAdCAM-1). Slides were incubated with these antibodies in an automatic immunostainer (Ventana Benchmark Ultra, Eindhoven, the Netherlands). After performing immunostaining, these slides were scanned with an Intellisite high-resolution scanner (Philips ultra-fast scanner 1.6 RA; Philips Digital Pathology, Best, The Netherlands) and analyzed within the IntelliSite Pathology Solution Image Management System (IMS, Philips Digital Pathology, Best, The Netherlands).

The most inflamed biopsy at each HE stained slide was selected using the Geboes score. In each patient, the surface of the most affected colonic biopsy slide and the area

of follicular tissue was determined on the same HE, CD3 and CD20 immunostained sections. The total surface area of the biopsy (including follicular and extrafollicular tissue) was measured in square millimetres (mm^2). Lymphoid follicles were optically counted and divided in primary and secondary follicles based on the presence or absence, respectively, of germinal centers besides B and T cell compartments. Absolute numbers of ERG^+, $PNAd^+$ and $MAdCAM\text{-}1^+$ venules were optically counted within the extra- and intra-follicular surface of the whole biopsy at 40x magnification by one analyst. The proportion of $PNAd^+$ and $MAdCAM\text{-}1^+$venules among all ERG^+ venules was displayed in percentages (% $PNAd^+$ venules/ERG^+venules and % $MAdCAM\text{-}1^+$ venules/ERG^+ venules). We also described the density of $PNAd^+$ and $MAdCAM\text{-}1^+$ venules computed by dividing the absolute number of venules by the biopsy surface (respectively $PNAd^+$ venules/mm^2 and $MAdCAM\text{-}1^+$ venules/mm^2) and the percentage of $PNAd^+$ venules also expressing $MAdCAM\text{-}1^+$ (% double positive venules/ERG^+ venules).

Multiplex Immunoassay

Additionally, we aimed to analyze CCL-19 and CXCL-13 in the local and systemic cytokine milieu of UC patients. CCL-19 is expressed on HEVs and in T-cell zones of SLOs and it is required for trafficking and positioning of T cells and dendritic cells within SLOs. CXCL-13, or BLC/BCA-1, is a chemokine expressed in B-cell follicles. It is suggested that these homeostatic chemokines (both CCL-19 and CXCL-13) contribute to the generation and organization of lymphoid neogenesis[12].

Following initial ileocolonoscopy, venous blood was obtained from 22 UC patients and 10 HC. Colonic biopsies taken during endoscopy from newly diagnosed UC patients and HC were kept in a phosphate-buffered saline solution at 2–8 °C and processed within eight hours. Specimens were pooled and finely minced in Hanks'/1% bovine serum albumin using a 70-mm gaze and spatula followed by Ficoll density gradient centrifugation. The single cell suspension was resuspended, after washing, and stimulated overnight with PMA/ionomycin to induce chemokine/cytokine excretion.

Multiplex immunoassays were performed at the MultiPlex Core Facility of the Center for Translational Immunology (UMC Utrecht, Utrecht, The Netherlands) using an in-house validated platform (ISO9001) on the serum samples and supernatants of the cultured biopsies as previously described[27]. Undetectable analyte results were replaced by the lowest measured value in the patient group divided by two. This surrogate value was always below the lower limit of detection.

Statistical Analysis

Continuous variables were expressed as mean with standard deviation (±SD) or as median with interquartile range (IQR) depending on skewness. Categorical data were expressed as numbers with percentage and analyzed using the Chi-square when necessary. To study change in expression of PNAd and MAdCAM-1 on colonic mucosal venules

over time, paired baseline and follow-up variables were compared using the paired T-test or Wilcoxon signed rank test depending on skewness, whereas continuous variables of unpaired groups were compared using the independent T-test or Mann-Whitney U test.

To determine if the variables age, gender, smoking behavior, symptom duration prior to initial diagnosis, location of UC, disease activity expressed using the Geboes score (severity stage for each separate Grade), effective remission induction treatment directly after diagnosis and treatment at follow-up were correlated with the expression of PNAd and MAdCAM-1 on colonic mucosal venules at diagnosis and during follow-up, a linear regression model was used. Factors with a *p*-value <0.2 in univariable analysis were included in a multivariable linear regression model with backward elimination. A two-sided p-value of 0.05 was considered to be statistically significant. Data analysis was performed using the SPSS statistical software (version 24.0.0.0; IBM Corp, Armonk, NY, USA) and GraphPad Prism (Graphpad Software version 7.0, La Jolla, CA, USA).

Ethics

The study protocol (NL28761.091.09) was approved by the research ethics committee of the Radboud University Nijmegen Medical Centre (CMO Regio Arnhem-Nijmegen, Nijmegen, The Netherlands). The procedures were performed in accordance with the Declaration of Helsinki (version 9, 19 October 2013).

Results

Baseline Characteristics Study Population

We included biopsy specimens of 378 untreated UC patients at diagnosis and during follow-up, and 10 HC. All patients had an endoscopic Mayo score of at least one at baseline colonoscopy. To reach remission after initial diagnosis, 223 (59%) UC patients required aminosalicylates, 42 (11.1%) needed topical steroids and the remaining patients were in need of oral steroids, anti-TNF treatment or resective surgery (*n* = 104, 27.6%). The baseline characteristics of all patients and HC are presented in **Table 1**.

Values expressed in n (%) or as median with interquartile range; HC, Healthy control; IBD, inflammatory bowel diseases; IQR, Interquartile range; UC, ulcerative colitis.

The Presence of PNAd$^+$ and MAdCAM-1$^+$ Venules in Colonic Biopsies at Baseline

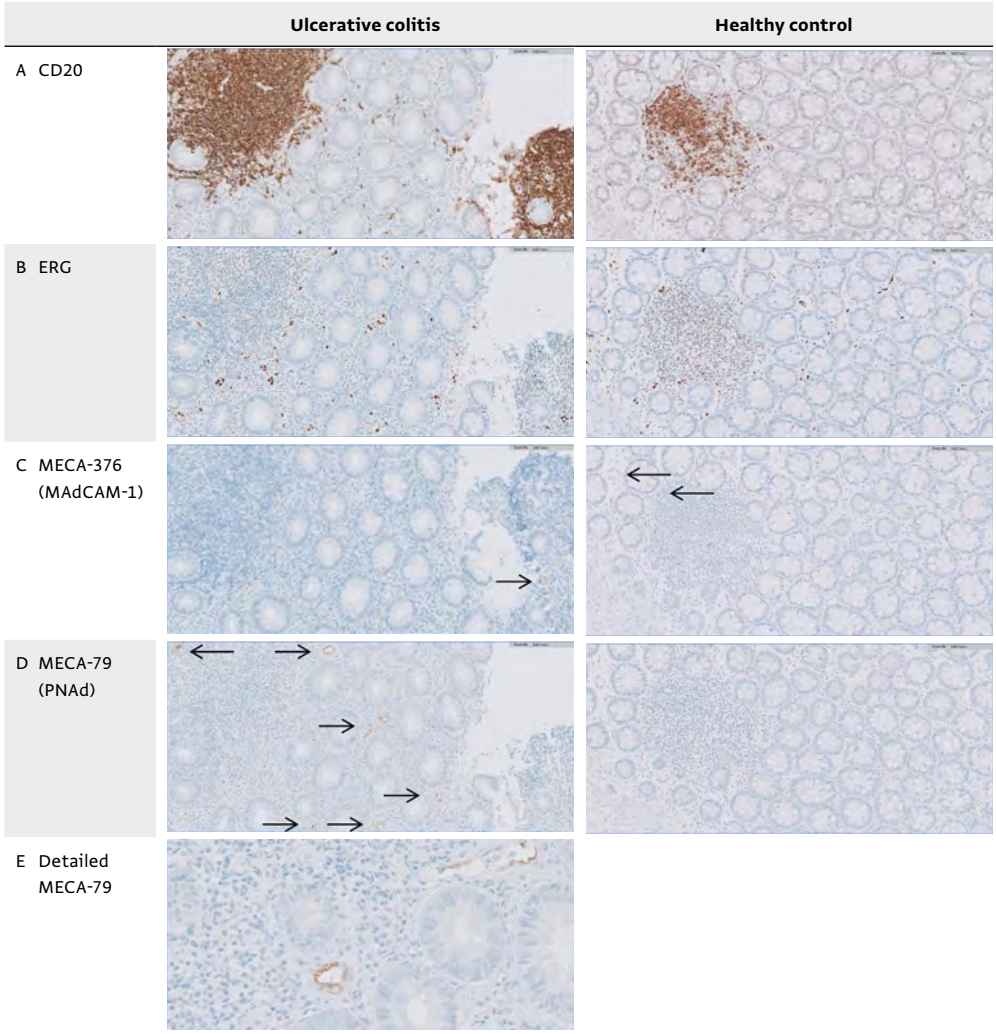
In the colonic mucosa of UC patients, higher numbers of follicles/mm^2 (median: 1.3 (IQR 0.7–2.1)) were present compared to HC (median: 0.04 (IQR 0.0–0.09), p = 0.001) (**Table 2**). Consequently, the total follicular surface per colonic biopsy was higher in UC (median 0.15 (0.05–0.32) mm^2) vs. HC (0.04 (0.0–0.09) mm^2, p = 0.004). All ERG$^+$ venules were

Table 1 *Baseline characteristics.*

	UC (*n* = 378)	HC (*n* = 10)
Age at Diagnosis in Years	43 (30–57)	36 (26–43)
Sex		
• Female	190 (50.3%)	6 (60%)
• Male	188 (49.7%)	4 (40%)
Extra-Intestinal Manifestations		
• Yes	46 (12.2%)	-
Family History of IBD		
• Yes	42 (11.1%)	-
Duration of Complaints before Diagnosis in Weeks	10 (4-16)	-
Smoking Status at Baseline Endoscopy		
• Ceased	84 (21.5%)	0 (0%)
• Yes	48 (12.3%)	3 (30%)
• No or unknown	246 (66.2%)	7 (70%)
Calprotectin	322 (167-1161)	-
Mayo Endoscopic Score		
• Mayo 0	-	10 (100%)
• Mayo 1	156 (41.3%)	0 (0%)
• Mayo 2	174 (46.0%)	0 (0%)
• Mayo 3	48 (12.7%)	0 (0%)
UC Localization		
• Extent	136 (36.0%)	-
○ E1: Ulcerative proctitis	149 (39.4%)	
○ E2: Left-sided UC	93 (24.6%)	
○ E3: Extensive UC		
Histological Inflammation Geboes Score		
• ≥ 0.1 and < 3.1	2 (0.5%)	10 (100%)
• ≥ 3.1	376 (99.5%)	0 (0%)
Location Biopsies Taken at Baseline Endoscopy		
• Rectum	143 (37.8%)	-
• Sigmoid	152 (40.2%)	-
• Right-sided	2 (0.5%)	-
• Left- and right-sided	81 (21.4%)	10 (100%)
Effective Remission Induction Treatment at Diagnosis		
• No treatment	9 (2.4%)	10 (100%)
• 5-ASA	223 (59.0%)	-
• Topical steroids	42 (11.1%)	-
• Oral steroids	86 (22.8%)	-
• Anti-TNF	15 (4.0%)	-
• Resective surgery	3 (0.8%)	-

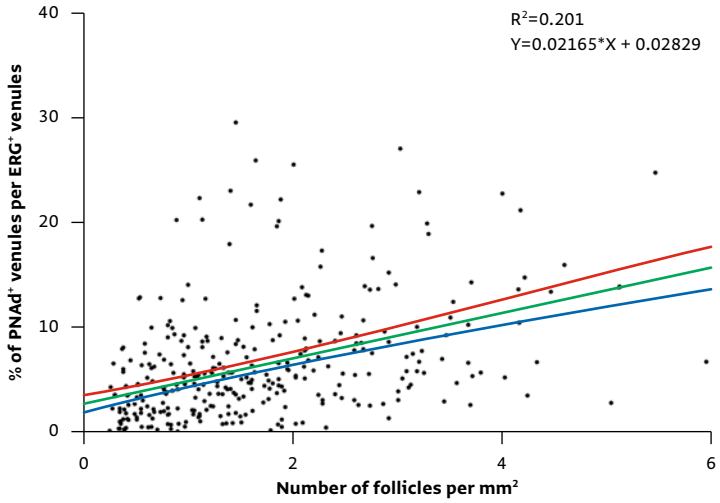
located outside these lymphoid follicles (i.e. extrafollicular). Likewise, the majority of PNAd$^+$ and MAdCAM-1$^+$ venules were found extrafollicular (**Figure 1**).

Figure 1 *Immunohistochemical staining indicating the presence of follicles and PNAd$^+$ and MAdCAM-1$^+$ venules in inflamed colonic tissue of UC patients and non-inflamed colonic tissue of a healthy control. Representative photomicrographs, with a magnification of x 20, of a colonic biopsy from a UC patient with (A) CD20 staining indicating B-cells (and follicles), (B) ERG staining indicating all venules, (C) MECA-367 staining indicating MAdCAM-1$^+$ venules (pointed out with black arrows), (D) MECA-76 staining indicating PNAd$^+$ venules (pointed out with black arrows) and (E) MECA-76 staining in detail with a magnification of x 40. Almost all PNAd$^+$ and MAdCAM-1$^+$ venules are located extrafollicular. MAdCAM-1, mucosal vascular addressin cell adhesion molecule-1; PNAd, peripheral node addressin; UC, Ulcerative colitis.*



At diagnosis, the median proportion of PNAd$^+$ venules was 4.9% (IQR 2.0-8.3%) in biopsies of UC patients with only 15 patients having none PNAd$^+$ venules. These PNAd$^+$ venules were completely absent in all HC (0.0 % (0.0-0.0%), p = 0.001) (**Figure 1**). The median proportion of MAdCAM-1$^+$ venules was also higher in UC patients at diagnosis (5.8% (IQR 2.5-9.9%)) compared to HC (0.8% (IQR 0.0-3.8%), p = 0.001). Of all ERG$^+$ venules, 1.6% (IQR 0.4-3.7%) were both MAdCAM-1$^+$ and PNAd$^+$, whereas 48.0% (IQR 13.9-69.4%) of all PNAd$^+$ venules were MAdCAM-1$^+$ as well. A correlation was found between the number of follicles per mm^2 biopsy and the relative number of PNAd$^+$ venules (**Figure 2**, *Rho* = 0.512, p = 0.001).

Figure 2 *Correlation between the number of follicles per mm^2 and the proportion of PNAd$^+$ venules per ERG$^+$ venules in the biopsies of inflamed UC. ERG, ETS related gene; PNAd, peripheral node addressin; UC, ulcerative colitis.*



The presence of PNAd$^+$ and MAdCAM-1 $^+$ venules in colonic biopsies at follow-up

Follow-up colonoscopy was performed after a median period of 40 months (IQR 14-85). At follow-up 25 % of 219 UC patients (n=93) were in endoscopic remission, while 75% (n=285) had endoscopic disease activity (Mayo ≥1). At follow-up endoscopy, 73.5% of all UC patients used immunosuppressive medication. Detail about endoscopic disease activity and disease location of the study population at follow-up can be found in **Supplementary Table S1**.

The median number of follicles/mm^2 measured at follow-up endoscopy in UC patients in endoscopic remission was significantly lower (0.4 (IQR 0.0-0.7)) compared to their own baseline values at diagnosis (1.1 (0.5-1.9), p = 0.001) resulting in a smaller

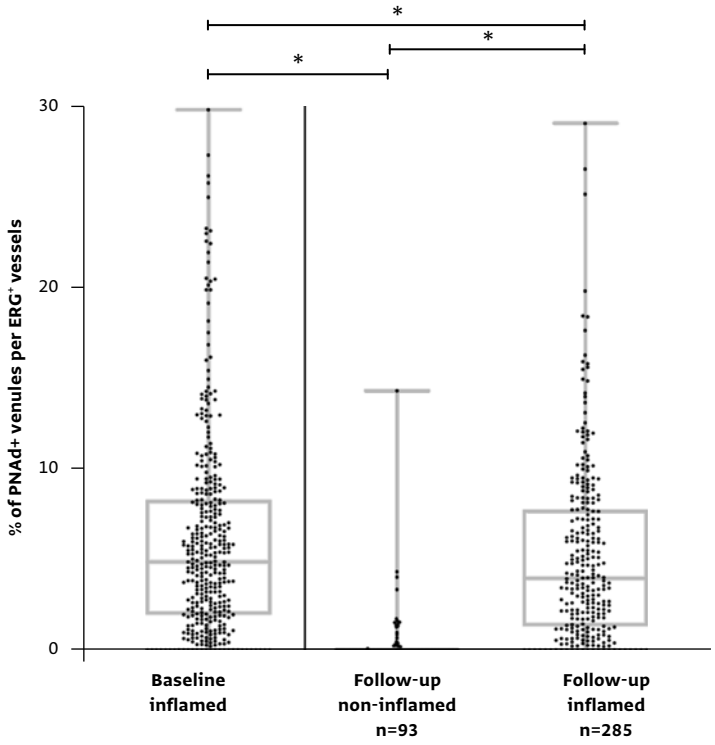
Table 2 *The presence of follicles and extrafollicular PNAd$^+$, MAdCAM-1$^+$ and/or ERG$^+$ venules in the inflamed colonic biopsies at UC diagnosis compared to healthy controls.*

	UC (Inflamed) *N* = 378	HC (Non-Inflamed) *N* = 10	*P*-value
Total Surface of the Biopsy in mm^2	2.3 (1.8-3.0)	2.1 (1.6-3.1)	*0.62*
Total Follicular Surface in mm^2	0.15 (0.05-0.32)	0.04 (0.0-0.09)	0.004
Total Extra Follicular Surface in mm^2	2.0 (1.5-2.7)	2.0 (1.6-3.1)	0.93
Follicles			
• Absolute number	3.0 (1.0-5.0)	1.0 (0.0-1.0)	0.001
• Number per mm^2	1.3 (0.7-2.1)	0.04 (0.0-0.09)	0.001
Extrafollicular ERG$^+$ Venules			
• Absolute number	366 (263-485)	206 (162-244)	0.001
• Number per mm^2 (density)	159 (130-193)	108 (66-116)	0.001
Extrafollicular PNAd$^+$Venules			
• Absolute number	(6.0-34.0)	(0.0-0.0)	0.001
• Number per mm^2 (density)	7.7 (2.6-14.0)	(0.0-0.0)	0.001
• As % of ERG$^+$ venules (proportion)	(2.0-8.3)	0.0 (0.0-0.0)	0.001
• Absolute Number of Intrafollicular PNAd$^+$ Venules	1.0 (0.0-4.0)	(0.0-0.0)	0.008
Extrafollicular MAdCAM-1$^+$Venules			
• Absolute number	19.0 (8.0-36.0)	1.5 (0.0-8.8)	0.001
• Number per mm^2 (density)	9.1 (3.8-15.6)	0.5 (0.0-3.9)	0.001
• As % of ERG$^+$ venules (proportion)	5.8 (2.5-9.9)	(0.0-3.8)	0.001
• Absolute Number of Intrafollicular MAdCAM-1$^+$ Venules	0.0 (0.0-3.0)	0.0 (0.0-1.5)	0.19
Extrafollicular MAdCAM-1$^+$ PNAd$^+$ Venules			
• Absolute number	6 (1.0-15.0)	0 (0-0)	0.001
• Number per mm^2 (density)	2.4 (0.6-6.0)	0 (0-0)	0.001
• As % of ERG$^+$ venules (proportion)	1.6 (0.4-3.7)	0 (0-0)	0.001
Percentage MAdCAM-1$^+$ of PNAd$^+$ Venules	42.9 (18.1-70)	*	-

total follicular surface (0.03 (IQR 0.0-0.1) mm^2 versus the median: 0.12 (0.03-0.3) mm^2, p = 0.001). The proportion of PNAd$^+$ venules in UC patients in endoscopic remission completely disappeared (0.0%(IQR 0.0-0.08%), **Figure 3**). In contrast, the proportion of MAdCAM-1$^+$venules remained the same when comparing patients in endoscopic remission at follow-up (7.8% (IQR 4.5-11.8%)) with their own baseline levels (6.9% (IQR 2.6-11.0%), p = 0.15).

UC patients with active endoscopic disease at follow-up demonstrated significant lower proportions of PNAd$^+$ venules (4.0% (IQR 1.3-7.8%)) compared to their own baseline levels (5.3% (2.2-8.3%), p = 0.04), whereas an upregulation of MAdCAM-1 from baseline (5.5% (IQR 2.5-9.5%)) to follow-up (7.8% (IQR 4.5-12.0%), p = 0.001) was demonstrated.

Figure 3 *The proportion of PNAd⁺ vessels per ERG⁺ vessels in UC patients at baseline and at follow-up. PNAd, peripheral node addressin; UC, ulcerative colitis. * p-value < 0.05.*



No difference in the proportion of MAdCAM⁺ venules was detected between patients in endoscopic remission and active disease during follow-up (p = 0.80) (**Table 3**).

Association of PNAd and MAdCAM-1 with Disease Phenotype at Diagnosis

The results of the linear regression analysis are shown in **Table 4**. The univariable analysis showed that male sex (β = 0.16, SE 0.006, p = 0.002), smoking (β = 0.13, SE 0.004, p = 0.01) and pancolitis (β = 0.35, SE 0.004, p = 0.001) were significantly associated with lower baseline proportions of PNAd⁺ venules in UC patients (**Table 4A**). Of note, the absolute number of PNAd⁺ venules was lower in smoking UC patients, while the number of ERG⁺ venules was comparable between smokers and non-smokers (p = 0.001).

In addition, a longer duration of symptoms before diagnosis (β = 0.25, SE 0.001, p = 0.001), histological disease activity, as indicated by the Geboes scores describing architectural changes (Geboes 0, β = 0.20, SE 0.004, p = 0.002), chronic inflammatory infiltrate (Geboes 1, β = 0.22, SE 0.006, p = 0.001) and the presence of erosions and ulcerations (Geboes 5, β = 0.13, SE 0.002, p = 0.05), and rectal disease activity (9 = 0.35,

Table 3 *The presence of follicles and (PNAd$^+$, MAdCAM-1$^+$, ERG$^+$) venules in inflamed and non-inflamed colonic biopsies of UC patients at follow-up endoscopy.*

	UC Patients in Remission at Follow-Up *n* = 93		
	Baseline Inflamed	**Follow-up Non-Inflamed**	***P*-value**
Total Surface of the Biopsy in mm^2	2.4 (1.7-3.2)	2.0 (1.5-2.7)	*0.10*
Total Follicular Surface in mm^2	0.12 (0.03-0.3)	0.03 (0.0-0.1)	*0.001*
Total Extra Follicular Surface in mm^2	2.1 (1.5-3.0)	2.0 (1.5-2.6)	*0.43*
Follicles			
• Absolute number	2 (1-4)	1 (0-1)	*0.001*
• Number per mm^2	1.1 (0.5-1.9)	0.4 (0.0-0.7)	*0.001*
ERG$^+$ Venules			
• Absolute number	336 (232-468)	235 (164-339)	*0.001*
• Number per mm^2 (density)	146 (123-173)	123 (93-155)	*0.001*
Extrafollicular PNAd$^+$ Venules			
• Absolute number	14 (3-34)	0.0 (0.0-0.8)	*0.001*
• Number per mm^2 (density)	6.0 (1.4-11.8)	0.0 (0.0-0.17)	*0.001*
• As % of ERG$^+$ venules (proportion)	4.3 (1.0-8.6)	0.0 (0.0-0.08)	*0.001*
Absolute number of intrafollicular PNAd$^+$ Venules	1 (0-3)	0.0 (0.0-0.0)	*0.001*
Extrafollicular MAdCAM-1$^+$ Venules			
• Absolute number	20 (8.5-42.0)	18 (9.0-32.0)	*0.38*
• Number per mm^2 (density)	10.7 (3.6-16.3)	10.0 (5.1-15.1)	*0.71*
• As % of ERG$^+$ venules (proportion)	(2.6-11.0)	7.8 (4.5-11.8)	*0.15*
Absolute number of intrafollicular MAdCAM-1$^+$ Venules	0.0 (0.0-2.0)	0.0 (0.0-1.0)	*0.02*
Extrafollicular MAdCAM-1$^+$ PNAd$^+$ Venules			
• Absolute number	5 (0-11)	0 (0-0)	*0.001*
• Number per mm^2 (density)	2.03 (0.0-4.52)	0 (0-0)	*0.001*
• As % of ERG$^+$ venules (proportion)	1.4 (0.0-2.7)	0 (0-0)	*0.001*
Percentage MAdCAM-1$^+$ of PNAd$^+$ venules	48.0 (13.9-69.4)	*	-

Values expressed in n (%) or as median (interquartile range); ERG, ETS related gene; HC, Healthy control; MAdCAM-1, mucosal vascular addressin cell adhesion molecule-1; PNAd, peripheral node addressin; UC, ulcerative colitis.

SE 0.004, p = 0.001) were significantly associated with higher baseline proportions of PNAd$^+$ venules in UC (**Supplementary Figure 1**). The age at diagnosis and Geboes scores describing the amount of eosinophils in the lamina propria (2A) and the presence of cryptitis (4) were not significant variables in univariable analysis, but were included in the multivariable analysis as the p-value was below the prespecified threshold of 0.2. There was no association between Geboes describing the number of neutrophils in the

UC Patients with Active Disease at Follow-Up *n* = 285			
Baseline Inflamed	**Follow-up Inflamed**	***P*-value**	***P*-value Follow-Up Non-Inflamed vs Inflamed**
2.3 (1.8-2.9)	2.2 (1.7-2.8)	0.21	0.39
0.16 (0.06-0.34)	0.1 (0.04-0.3)	0.001	0.001
2.0 (1.6-2.6)	2.0 (1.6-2.6)	0.92	0.60
3 (1-5)	2 (1-4)	0.001	0.001
1.4 (0.7-2.2)	1.1 (0.5-1.8)	0.001	0.001
371 (274-490)	394 (292-546)	0.12	0.001
163 (131-194)	184 (150-224)	0.001	0.001
19 (7-35)	17 (5-32)	0.39	0.001
8.3 (3.4-14.0)	7.7 (2.8-14.3)	0.85	0.001
5.3 (2.2-8.3)	4.0 (1.3-7.8)	0.04	0.001
2 (0-5)	0 (0-3)	0.003	0.001
18 (8-35)	32.0 (16.0-52.0)	0.001	0.001
9.0 (3.8-15.5)	14.2 (8.4-22.2)	0.001	0.001
5.5 (2.5-9.5)	7.8 (4.5-12.0)	0.001	0.80
0 (0-3)	0.0 (0.0-3.0)	0.37	0.002
6 (1-15)	8 (2-17)	0.04	0.001
2.5 (0.7-6.4)	3.6 (0.8-7.8)	0.002	0.001
1.8 (0.4-3.8)	2.0 (0.5-3.9)	0.21	0.001
42.9 (19.1-70.5)	57.9 (35.7-83.3)	0.001	-

* There are no PNAd$^+$ venules in non-inflamed biopsies, therefore no percentage is presented.

lamina propria (2B) and epithelium (3) and the proportion of PNAd$^+$ venules. Multivariable analysis identified symptom duration before diagnosis (p = 0.01), disease location (p = 0.001) and Geboes 0 (p = 0.01) as independent variables associated with the proportion PNAd$^+$ venules. A follow-up endoscopy, disease location was also an independent factor associated with the proportion of PNAd$^+$ venules (p = 0.001, **Supplementary Table 2A**).

Table 4 *Univariable and multivariable analysis of potential variables correlating with the expression of PNAd$^+$ (Table 4A) and MAdCAM-1$^+$ (Table 4B) venules as a proportion of ERG$^+$ venules at diagnosis.*

A Baseline PNAd		Univariable		Multivariable	
	R2 (%)	β (SE)	p-value	β (SE)	p-value
Age at diagnosis	0.7	−0.08 (0.001)	0.11		NS
Sex*	2.6	−0.16 (0.006)	0.002		NS
Smoking behavior**	1.8	−0.13 (0.004)	0.01		NS
Symptom duration prior to initial diagnosis in weeks	6.1	0.25 (0.001)	0.001	0.22 (0.001)	0.01
Disease location º	12.4	−0.35 (0.004)	0.001	-0.40 (0.005)	0.001
Histologic disease activity					
Geboes 0	3.9	0.20 (0.004)	0.002	0.21 (0.005)	0.01
Geboes 1	4.7	0.22 (0.006)	0.001	-	NS
Geboes 2A	1.0	0.10 (0.005)	0.11	-	NS
Geboes 2B	0.2	0.05 (0.004)	0.44		NS
Geboes 3	0	0.02 (0.005)	0.73		NS
Geboes 4	1.1	0.11 (0.008)	0.10		
Geboes 5	1.6	0.13 (0.002)	0.05		
First effective remission induction treatmentºº	1.4	-0.12 (0.002)	0.02		NS
Number exacerbations per year clinical follow-up	0.8	0.09 (0.015)	0.08		NS

*Female = 0 , male = 1 **Never = 0, ceased = 1, yes = 2 ºProctitis = 1, left-sided = 2, extensive = 3, ººNo treatment = 0, 5ASA = 1, topical steroids = 2, oral steroids = 3, anti-TNF = 4, anti-integrins = 5, surgery = 6; β = Regression coefficient, SE = Standard Error.

All subdomains of the Geboes score were associated with lower baseline proportions of MAdCAM-1$^+$ venules in UC patients with p-values below 0.2 in the univariable analysis (**Table 4B**). In multivariable analysis, this association was not statistically significant.

Association of PNAd$^+$ and MAdCAM-1$^+$ Venules with Disease Course

Patients who responded to aminosalicylates and/or topical steroids (n = 265, 70%) as initial remission induction therapy displayed a higher proportion of PNAd$^+$ venules in baseline biopsies compared to patients in need of oral steroids, anti TNF treatment or surgery (n = 104, 27.6%, p = 0.02). The proportion of MAdCAM-1$^+$ venules at baseline did not distinguish responders to aminosalicylates and/or topical steroids from responders to oral steroids, anti-TNF and/or surgery.

Furthermore, in patients with active disease at time of follow-up endoscopy, we studied the association between current treatment and the proportion of PNAd$^+$ and MAdCAM-1$^+$ venules. The use of oral steroids and/or anti-TNF during follow-up was as-

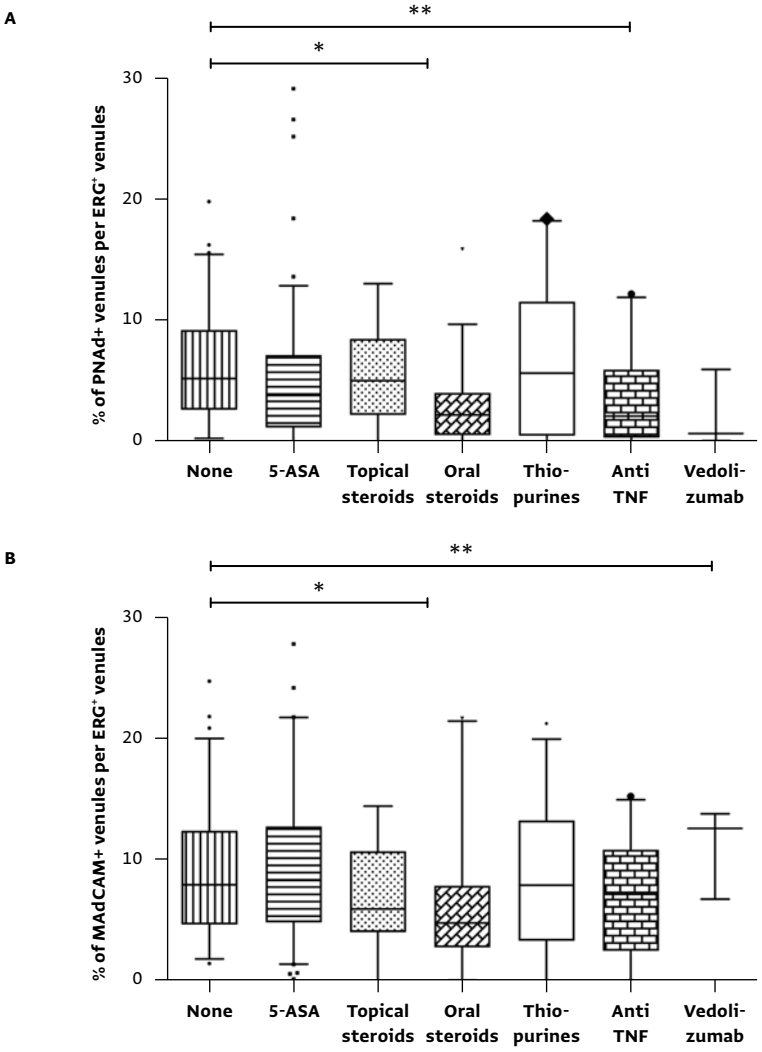
B Baseline MAdCAM-1		Univariable		Multivariable	
	R2 (%)	β (SE)	p-value	β (SE)	p-value
Age at Diagnosis	0.2	0.04 (0.001)	0.46		
Sex*	0.3	−0.05 (0.006)	0.34	-	-
Smoking Behavior**	0.3	0.05 (0.004)	0.35	-	-
Symptom Duration Prior to Initial Diagnosis in Weeks	0.3	0.06 (0.001)	0.44	-	-
Disease Location º	0	−0.01 (0.004)	0.84	-	-
Histologic Disease Activity					
Geboes 0	1.3	−0.11 (0.004)	0.07		NS
Geboes 1	1.3	−0.11 (0.006)	0.08		NS
Geboes 2A	3.1	−0.18 (0.005)	0.01		NS
Geboes 2B	2.4	−0.15 (0.004)	0.02		NS
Geboes 3	3.4	−0.19 (0.005)	0.003		NS
Geboes 4	1.2	−0.11 (0.005)	0.08		NS
Geboes 5	1.3	−0.11 (0.002)	0.08		NS
First Effective Remission Induction Treatment ºº	0.3	−0.06 (0.002)	0.29	-	-
Number Exacerbations Per Year Clinical Follow-Up	0.6	0.08 (0.187)	0.14		NS

*Female = 0, male = 1 **Never = 0, ceased = 1, yes = 2, ºProctitis = 1, left-sided = 2, extensive = 3, ººNo treatment = 0, 5ASA = 1, topical steroids = 2, oral steroids = 3, anti-TNF = 4, anti-integrins = 5, surgery = 6; β = Regressioncoefficient, SE = Standard Error.

sociated with a significantly lower proportion of $PNAd^+$ venules compared to patients with active disease who did not use any medication at that time. In addition, a significant decline in the proportion of $MAdCAM\text{-}1^+$ venules was found in patients using oral steroids during follow-up. Treatment with Vedolizumab was associated with an increased proportion of $MAdCAM\text{-}1^+$ venules in active disease during follow-up (**Figure 4**).

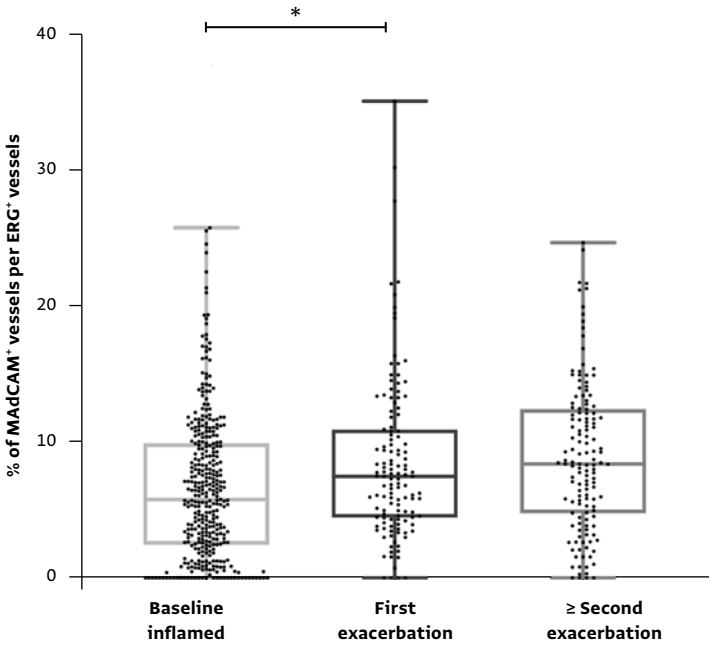
After initial remission, 89% of UC patients (*n* = 337) experienced at least one relapse, while 11% (*n* = 41) did not experience an exacerbation. The median number of clinical, biochemical and/or endoscopic confirmed exacerbations was two (IQR 1–3) during 128 months (IQR 85–165) of follow-up (which is equal to 0.18 (IQR 0.09–0.3) exacerbations per patient-year). The first exacerbation occurred after a median period of 25 months (IQR 10–59). When comparing patients with the highest proportion $MAdCAM\text{-}1^+$ venules (75th percentile, ≥ 9.9%) with patients with the lowest proportions (25th percentile, ≤ 2.5%) at baseline endoscopy, we found that patients with initial higher numbers of

Figure 4 *The effect of treatment during follow-up endoscopy on the percentages of (A) PNAd$^+$ venules and (B) MAdCAM-1$^+$ venules per ERG$^+$ venules.* p-value < 0.001; ** p-value < 0.05; 5-ASA, aminosalicylates; MAdCAM-1, mucosal vascular addressin cell adhesion molecule-1; PNAd, peripheral node addressin; UC, ulcerative colitis.*



MAdCAM-1$^+$ venules experienced significantly more exacerbations per year (0.24 (IQR 0.15-0.33) versus 0.17(0.08-0.32), p = 0.03). During a first exacerbation, the proportion of MAdCAM-1$^+$ venules was higher compared to baseline levels (respectively 7.5% (4.4-10.9%) and 5.8% (2.6-10.0%), p = 0.001). In case of a following exacerbation, the propor-

Figure 5 *The upregulation of the proportion of MAdCAM-1$^+$ vessels per ERG$^+$ vessels in UC patients during exacerbations. MAdCAM-1, mucosal vascular addressin cell adhesion molecule-1; UC, ulcerative colitis.* p-value < 0.05.*



tion of MAdCAM-1$^+$ venules (8.5% (5.0–12.5%)) was even higher compared to the proportions during the first exacerbation (**Figure 5**). In contrast, there was no upregulation of proportions PNAd$^+$ venules during subsequent flares.

CXCL-13 and CCL-19 in Serum and Stimulated Biopsies

In 22 of the included UC patients and 10 HC, the chemokines CXCL-13 and CCL-19 were analyzed in the serum and supernatants of stimulated biopsies which were collected during baseline endoscopy. In the serum of UC patients, we demonstrated statistically significant higher levels of CXCL-13 and CCL-19 compared to HC (CXCL-13: 86.2 pg/mL (IQR 58.3–109.4) versus 36.9 (IQR 31.3–48.8), p = 0.001; CCL-19: 167.7 pg/mL (IQR 133.8–204.3) versus 100.3 (IQR 37.4–158.1), p = 0.02). In the supernatant of the stimulated biopsies from UC patients, statistically significant higher levels of CCL-19 were found compared to HC (16.4 pg/mL (IQR 13.4–17.2) versus 10.5 (IQR 9.4–12.9), p = 0.001). The levels of CXCL-13 in the supernatant were also higher in UC patients compared to HC, but without reaching statistical significance (**Table 5**).

The number of follicles/mm^2 biopsy was correlated with the CXCL-13 level in serum (*Rho* = 0.47, p = 0.01) and the CCL-19 level in the supernatant (*Rho* = 0.43, p = 0.02), suggest-

Table 5 *Values of CXCL-13 and CCL-19 in the serum and supernatant of patient with ulcerative colitis at first presentation, compared with healthy controls (in pg/ml).*

	Inflamed UC *n* = 22	Healthy Controls n = 10	*p*-value
Serum in pg/mL			
CXCL-13	86.2 (58.3–109.4)	36.9 (31.3–48.8)	0.001
CCL-19	167.7 (133.8–204.3)	100.3 (37.4–158.1)	0.02
Supernatant Biopsy in pg/mL			
CXCL-13	4.3 (0.6–42.72)	0.6 (0.6–20.8)	0.363
CCL-19	16.4 (13.4–17.2)	10.5 (9.4–12.9)	0.001

ing a potential role for these chemokines in TLO formation. The density of MAdCAM-1$^+$ venules was related to the amount of CCL-19 in the supernatant (*Rho* = 0.53, *p* = 0.003). CCL-19 in the supernatant also correlate with the absolute number and density of PNAd$^+$ venules (per mm^2) (*Rho* = 0.41, *p* = 0.03 and *Rho* = 0.39, *p* = 0.04).

Discussion

In a large cohort of untreated UC patients at diagnosis we found a high proportion of colonic mucosal PNAd$^+$ and MAdCAM-1$^+$ venules (nearly all located outside lymphoid follicles). In HC, PNAd$^+$ venules were completely absent and MAdCAM-1$^+$ venules were present in very low numbers. The proportion of PNAd$^+$ HEVs was correlated with more follicles and a greater follicular surface. PNAd$^+$ HEVs were absent in the colonic mucosa of UC patients when no inflammation was present at follow-up. Simultaneously, in remission the number of follicles shifted to lower levels comparable to HC, while the proportion of MAdCAM-1$^+$ venules remained unchanged. On the other hand, patients with active endoscopic disease at follow-up were found to have a comparable proportion of PNAd$^+$ HEVs to levels at diagnosis, while a further increase in the proportion of MAdCAM-1$^+$ venules was found with each subsequent exacerbation.

In line with our results, several reports have shown that PNAd$^+$ HEVs are never found in the lamina propria of the intestine of healthy controls.[27,28] These PNAd$^+$ HEVs were only found in Peyers patches, the appendicular mucosa and in mesenteric lymph nodes, but with a less intense staining compared to PNAd$^+$ HEVs found in tonsils and peripheral lymph nodes.[27,28]

Our findings of PNAd$^+$ venules being present in the colonic mucosa during inflammation of UC are in line with two earlier studies performed in a small patient group.[14,15] However, these studies did not take MAdCAM-1 positivity into account, and were cross-sectional in design providing no information during the follow-up of the disease. Expression of PNAd on mucosal HEVs in active disease enables the recruitment of T cells to TLOs.[11] Results from the previous studies using different methods (flowcytometry versus immunohistochemistry) suggest the recruitment of L-selectin-expressing naive T cells through these PNAd$^+$ HEVs.[14,15] Categorizing patients into groups based on the relative number of PNAd$^+$ venules (HEVhigh and HEVlow), a correlation was found between the presence of more lymphoid follicles and higher percentages of Tn and Tcm lymphocytes in the HEVhigh group.[14] In the present study, we demonstrated a statistically significant correlation between the proportion of PNAd$^+$ venules and the number of lymphoid follicles. In HC, the PNAd$^+$ venules are exclusively found in SLOs and absent in extrafollicular mucosal tissue. The presence of these extrafollicular PNAd$^+$ HEVs in active UC suggests formation of newly formed TLO in the chronic inflamed tissue thereby controlling the influx and potential local activation of T cells. We demonstrate that PNAd$^+$ HEVs disappear in UC patients with inactive disease at follow-up endoscopy. Therefore, these venules can be described as a marker of disease activity in UC.

In HC, MAdCAM-1 is exclusively expressed on endothelium of flattened venules in the lamina propria along the gastrointestinal tract and in associated lymphoid tissue, and it is not detected in extra-intestinal tissues, including those with mucosal surfaces.[8] This is in line with our results in the biopsies of HC. The available literature in long-standing UC patients describes increased numbers of MAdCAM-1$^+$ venules compared to the healthy control.[8,16,29–31] We demonstrated comparable increased numbers of MAdCAM-1$^+$ venules in newly diagnosed UC. In accordance with the present results, other studies showed no statistically significant difference between arbitrary chosen active and remission phases for the number of MAdCAM-1$^+$ venules in different UC patients during follow-up. We describe a statistically significant upregulation of the proportion of MAdCAM-1$^+$ venules over time, from baseline to the first exacerbation within the same patient. After experiencing several exacerbations, the number of MAdCAM-1$^+$ venules increase to even higher levels compared to those found at the moment of the first exacerbation. The MAdCAM-1 levels in patients who never experienced an exacerbation after diagnosis remain comparable to levels at diagnosis. In primary cultures of human intestinal microvascular endothelial cells, MAdCAM-1 gene and protein expression is induced in response to inflammatory cytokines (TNFα and IL-1β) and bacterial endotoxin (LPS) requiring both NF-κB and PI3-K/Akt activation.[32] Longer culture duration and higher cellular densities of microvascular endothelial cells (suggesting that cell-cell interaction plays a critical role) lead to more MAdCAM-1 expression induced by TNFα or LPS stimulation.[32] A longer total disease duration (time of all exacerbations added together) resulted

in more exposure to these inflammatory cytokines and can possibly explain the upregulation of MAdCAM-1 over time after experiencing more exacerbations.

Besides HEVs and flattened venules, different chemokines and cytokines play an important role in controlling the migration of T cells to the intestine. Homeostatic chemokines such as CXCL-13 and CCL-19 and lymphoneogenic cytokines like lymphotoxin αβ are involved in the recruitment of peripheral T cells to the site of inflammation where newly developed tertiary lymphoid organs are formed.[12,33] In the serum and supernatant of stimulated biopsies from 22 UC patients we found an upregulation of both CXCL-13 and CCL-19 compared to healthy controls. Higher levels of CXCL-13 in the serum and CCL-19 in the supernatant of biopsies were correlated with the presence of more follicles/mm² in the inflamed gut mucosa. Higher densities of PNAd$^+$ HEVs and MAdCAM-1$^+$ venules were also associated with higher CCL-19 in the supernatant. These findings suggest a possible role of CXCL-13 and CCL-19 in PNAd$^+$ and MAdCAM-1$^+$ expression and TLO formation.[11,12,33–35] The use of these chemokines as targets may be limited because similar molecular mechanisms are involved in the formation and maintenance of secondary and tertiary lymphoid organizations. Blocking CXCL-13 or CCL-19 could potentially harm lymphoid follicles disseminated throughout the whole body.[35]

The presence of large aggregates of lymphocytes with T/B cell compartmentalization and PNAd$^+$ HEVs (indicating lymphoid neogenesis) in the synovial fluid of patients with rheumatoid arthritis (RA) has been associated with disease severity, the frequency of exacerbations and an inferior response to frontline biological therapies that target TNF.[36] Therapeutic reversal of these lymphoid neogenesis features was correlated with good clinical responses in RA, but it remains unclear whether newly formed lymphoid follicles (TLOs) indicate distinct disease phenotypes or an evolutionary manifestation of chronic inflammation.[37] In our UC cohort, we found that higher proportions of PNAd$^+$ venules at baseline endoscopy were associated with longer symptom duration before diagnosis, histologic more severe disease activity as indicated by the Geboes score describing architectural changes and rectal disease activity. The correlation of higher proportions of PNAd$^+$ HEVs with longer duration of complaints before diagnosis suggests that PNAd$^+$ HEVs are a reflection of chronicity. Lower proportions of PNAd$^+$ venules were correlated with male sex, smoking and extensive colitis. The correlation between higher numbers of PNAd$^+$ HEVs in baseline biopsies and initial response on 5-ASA therapy and local steroids in our retrospective cohort should be interpreted with caution. At this point, a relation between PNAd expression and the working mechanism of 5-ASA therapies on mucosal inflammation is not known. The effect of medication and its relation to PNAd expression should be further studied using a prospective study design. When comparing our results in UC patients with findings in RA, we can confirm a similar role for PNAd$^+$ HEVs in assessing disease activity.

A higher proportion of MAdCAM-1$^+$ venules at baseline was associated with more exacerbations during clinical follow-up. Given the upregulation of MAdCAM-1 over time, based on the number of exacerbations, this supports the hypothesis that treatment with antibodies against MAdCAM-1(Ontamalimab) could be effective in inducing and maintaining remission in patients with longstanding disease (activity).[19]

It is well known that smoking ameliorates UC and smoking cessation has been associated with the onset of UC.[38,39] Nicotine has been considered to be responsible for the protective effect of smoking in UC, while the exact mechanism of action remains unclear. The research group of Maruta demonstrated in vivo that nicotine suppresses the increased recruitment of leukocytes. This was explained by a decrease of MAdCAM-1 expression on endothelial cells in the gut of DSS-induced colitis, possibly through a direct effect of nicotine on the vascular endothelium.[40,41] In contrast, our results indicate that smoking leads to reduced proportions of PNAd$^+$ venules with no difference in the numbers of MAdCAM-1$^+$ venules between smokers and non-smokers. The reduction in PNAd$^+$ venules can lead to a decreased influx of naive T cells to the gut, which has been associated with less severe inflammation.[42]

At the moment, there is an urgent need for biomarkers predicting response prior to treatment initiation as different treatment options are not effective in all patients.[43] For instance, pre-treatment analysis of α4β7 expression on lymphocytes in blood of IBD patients could be of value in predicting response on anti-α4β7 antibodies.[44,45] We showed that PNAd$^+$ venules contribute in predicting response on initial induction therapy. Since PNAd$^+$ venules are also presented in lymphoid tissue outside the gastrointestinal tract, blocking them might have harmful side effects.[46] UC patients with higher numbers of PNAd$^+$ HEVs, associated with more Tn- and fewer α4β7$^+$ Tem cells,[14] might benefit less of treatment with anti-α4β7$^+$ antibodies and/or anti-MAdCAM-1 treatment. MAdCAM-1$^+$ venules might be a better therapeutical target compared to PNAd$^+$ venules due to their gut-specific presence and upregulation in time during active inflammation. Patients with higher numbers of MAdCAM-1$^+$ venules, suggesting more recruitment of α4β7$^+$ T cells, might benefit more from anti-MAdCAM-1 treatment.

The strength of the present study is that it comprises a large cohort of newly diagnosed UC patients with serial measurements during remission and active disease phases, revealing the evolution in time of these vascular adressins. Further prospective research should be undertaken to investigate the value of PNAd$^+$ and MAdCAM-1$^+$ venules as treatment targets, biomarkers in predicting response to therapy and the effect of different treatments on the development and function of these venules.

In conclusion, a new formation of mucosal PNAd$^+$ HEVs is associated with the presence and histologic severity of inflammation and therefore a potential marker for disease activity in UC. Higher proportions of PNAd$^+$ HEVs were associated with more

colonic follicles, suggesting formation of tertiary lymphoid organs in the inflamed gut mucosa. As a high proportion of MAdCAM-1$^+$ venules is predictive for the number of exacerbations and increases with each subsequent exacerbation, prospective studies should focus on its possible predictive role and value as a therapeutic target.

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Supplementary materials

Table S1 *Patient characteristics at follow-up endoscopy.*

	All UC patients n=378	Non-inflamed UC at follow-up n=93	Inflamed UC at follow-up n= 285	p-value**
Clinical follow-up time in months	128 (85-165)	150 (106.5-169.5)	121 (82-159)	0.002
Time till first exacerbation in months				
• No exacerbation during follow-up, n (%)	25 (10-59)	48 (18-125)	21 (9-49)	0.001
• Exacerbation, n (%)	41 (10.8%)	28 (30.1%)	13 (4.6%)*	
	337 (89.2%)	65 (69.9%)	272 (95.4%)	
Surgery during clinical follow-up	24 (6.3%)	2 (2.2%)	22 (7.7%)	0.06
Mayo endoscopic score				0.001
• Mayo 0	93(24.6%)	93(100%)	-	
• Mayo 1	107 (28.3%)	-	107 (37.5%)	
• Mayo 2	124 (32.8%)	-	124 (43.5%)	
• Mayo 3	54 (14.3%)	-	54 (18.9%)	
UC localization				
• Extent	86 (22.8%)	-	86 (30.2%)	0.001
• E1: Ulcerative proctitis	130 (34.4%)	-	130 (45.6%)	
• E2: Left-sided UC	69 (18.3%)	-	69 (24.3%)	
• E3: Extensive UC	93 (24.6%)	93 (100%)	-	
• Remission				
Histological inflammation Geboes score				
• ≥0.1 and <3.1	151 (43%)	80 (86%)	71 (25%)	0.001
• ≥3.1	217 (57%)	13 (14%)	214 (75%)	
Treatment at follow-up endoscopy				
• None	100 (26.5%)	21 (22.6%)	79 (27.7%)	0.002
• 5-ASA	152 (40.2%)	44 (47.3%)	108 (37.9%)	
• Topical steroids	14 (3.7%)	1 (1.1%)	13 (4.6%)	
• Oral steroids	37 (9.8%)	1 (1.1%)	36 (12.6%)	
• Immunomodulators	40 (10.6%)	16 (17.2%)	24 (8.4%)	
• Anti-TNF	32 (8.5%)	10 (10.8%)	22 (7.7%)	
• Anti-integrins	3 (0.8%)	0 (0%)	3 (1.1%)	
Highest treatment during follow-up				
• None	1(0.3%)	0 (0%)	1 (0.4%)	0.06
• 5-ASA	88 (22.7%)	25 (26.9%)	63 (22.1%)	
• Topical steroids	52 (13.4%)	8 (8.6%)	44 (15.4%)	
• Oral steroids	42 (10.8%)	11 (11.8%)	31 (10.9%)	
• Immunomodulators	101 (26%)	25 (26.9%)	76 (26.7%)	
• Anti-TNF	62 (16%)	22 (23.7%)	40 (14.0%)	
• Anti-integrins	9 (2.3%)	0 (0%)	9 (3.2%)	
• Surgery	23 (5.9%)	2 (2.2%)	21 (7.4%)	

Values expressed in n (%) or as median with interquartile range.

* Biopsies were taken during follow-up endoscopy in UC patients who did not reach remission after initial diagnosis.

** Non-inflamed UC compared to inflamed UC patients; HC, Healthy control; MAdCAM-1, mucosal vascular addressin cell adhesion molecule-1; PNAd, peripheral node addressin; UC, ulcerative colitis.

Figure S1 *Grades 0 (Architectural changes), 1 (Chronic inflammatory infiltrate) and 5 (The presence of erosions and ulcerations) of the Geboes score for assessment of ulcerative colitis histological disease activity correlate with % of PNAd⁺ venules per ERG⁺ venules. There was no significant association between the Geboes Grades 2 (Eosinophils and neutrophils in the lamina propria), 3 (Neutrophils in the epithelium) and 4 (Crypt destruction) and the proportion of PNAd⁺ venules (not shown in this Figure).* p-value < 0.05. ERG, ETS related gene; PNAd, peripheral node addressin; UC, ulcerative colitis.*

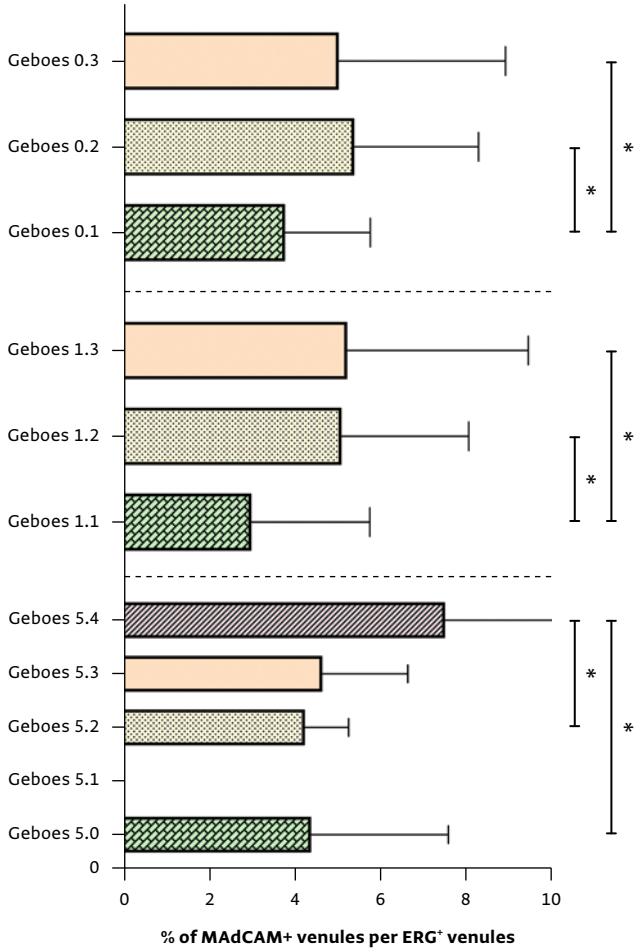


Table S2 *Univariate and multivariate analysis of potential variables correlating with the expression of PNAd$^+$ and MAdCAM-1$^+$ venules as a proportion of ERG$^+$ venules in active disease at follow-up endoscopy (A$^+$B).*

A Follow-up PNAd	Univariate			Multivariate	
	R2 (%)	β (SE)	p-value	β (SE)	p-value
Follow-up PA is the first exacerbation*	3.2	-0.18 (0.005)	0.08		NS
Disease location **	33.5	-0.58 (0.002)	0.001	-0.37 (0.002)	0.001
Histologic disease activity					
• Geboes 0	19.2	0.44 (0.004)	0.001	0.33 (0.006)	NS
• Geboes 1	21.7	0.47 (0.004)	0.001		NS
• Geboes 2A	19.1	0.44 (0.004)	0.001		NS
• Geboes 2B	27.4	0.52 (0.003)	0.001		NS
• Geboes 3	29.2	0.54 (0.005)	0.001		NS
• Geboes 4	32.0	0.57 (0.005)	0.001		0.003
• Geboes 5	17.8	0.42 (0.003)	0.001		NS

*Yes=1, no=2 ** Proctitis=1, left-sided=2, extensive=3
β=Regressioncoefficient, SE=Standard Error

B Follow-up MAdCAM-1	Univariate			Multivariate	
	R2 (%)	β (SE)	p-value	β (SE)	p-value
Follow-up PA is the first exacerbation*	5.6	-0.24 (0.012)	0.02		NS
Disease location**	0.1	-0.04 (0.006)	0.71	-	-
Histologic disease activity					
• Geboes 0	1.0	0.10 (0.011)	0.34	-	-
• Geboes 1	4.5	0.21 (0.01)	0.04	0.26 (0.01)	NS
• Geboes 2A	6.9	0.26 (0.01)	0.01	-	0.01
• Geboes 2B	5.3	0.23 (0.01)	0.02		NS
• Geboes 3	2.7	0.16 (0.013)	0.11		NS
• Geboes 4	1.7	0.13 (0.014)	0.20		NS
• Geboes 5	0.6	0.08 (0.009)	0.47		-

*Yes=1, no=2 ** Proctitis=1, left-sided=2, extensive=3
β=Regressioncoefficient, SE=Standard Error

CHAPTER 3

Mucosal α4β7^{+} lymphocytes and MAdCAM^{+} venules predict response to Vedolizumab in ulcerative colitis

INFLAMMATORY BOWEL DISEASE 2023 JUL 12;ONLINE AHEAD OF PRINT

Britt Roosenboom

Peter J. Wahab

Carolijn Smids

Jos Meijer

Larissa G.J.M. Kemperman

Marcel J.M. Groenen

Ellen G. van Lochem

Carmen S. Horjus Talabur Horje

Abstract

BACKGROUND Therapeutic strategies for patients with Ulcerative Colitis (UC) are based on patient and disease related factors in combination with drug characteristics, but fail to predict success in individual patients. A considerable proportion of UC patients do not respond to the biological Vedolizumab. Therefore pre-treatment biomarkers for therapeutic efficacy are urgently needed. Mucosal markers related to the integrin-dependent T-lymphocyte homing could be potent predictors.

METHODS We prospectively included 21 biological and steroid naive UC patients with moderate to severe disease activity planned to escalate therapy to Vedolizumab. At week 0, before initiating treatment, colonic biopsy specimens were obtained for immunophenotyping and immunohistochemistry. Clinical and endoscopic disease activity were determined at week 16 after four infusions of Vedolizumab. In addition, we retrospectively included 5 UC patients who were first treated with anti-TNFα before receiving Vedolizumab to compare with biological naïve patients.

RESULTS Abundance of $\alpha4\beta7$ on more than 8% of all $CD3^+$T lymphocytes in colonic biopsies at baseline was predictive for responsiveness to Vedolizumab (sensitivity 100%, specificity 100%). The threshold for the proportion of MAdCAM-1^+ and $PNAd^+$ of all venules in the biopsies predictive for responsiveness to Vedolizumab was ≥ 2.59 % (sensitivity 89%, specificity 100%) and ≥ 2.41% (sensitivity 61%, specificity 50%), respectively. At week 16, a significant decrease of $\alpha4\beta7^+CD3^+$T lymphocytes was demonstrated in responders (18% (12-24) to 8% (3-9), p=0.002), where no difference was seen in non-responders (4% (3-6) to 3%, p=0.59).

CONCLUSION UC responders to Vedolizumab have a higher percentage of $\alpha4\beta7^+CD3^+$T lymphocytes and a higher proportion of MAdCAM-1^+ venules in colonic biopsies than non-responders before initiating therapy. Both analyses could be promising predictive biomarkers for therapeutic response and may lead to more patient tailored treatment in the future.

Introduction

In daily practice, selection of specific therapeutic options for patients with chronic inflammation in Ulcerative Colitis (UC) is based on factors concerning drug characteristics (e.g. treatment adherence, presence of antidrug antibodies, drug clearance, safety profile) and disease-related factors (e.g. severity of disease, extraintestinal manifestations, comorbidity). With multiple (expensive) biologicals as treatment options available and a considerable proportion of patients who do not respond to treatment, we are in urgent need of biomarkers that better predict the responsiveness to drug therapy in the individual patient.[1] In this study we focus on potential biomarkers for responsiveness to the α4β7 integrin inhibitor Vedolizumab.

The GEMINI trials suggest that patients who would profit most from treatment with Vedolizumab, are patients with clinically and endoscopically less severe disease.[2-4] Exposure to Vedolizumab after failing anti TNFα treatment seemed to be less beneficial compared to the effect seen in biological naive patients.[5] Additionally, elevated inflammatory markers (such as CRP and fecal calprotectin) and changes in microbiome-diversity were associated with significant differences in response rates. These non-specific markers do not clarify why Vedolizumab lacks effect in a significant proportion of patients. Deeper insight in immune mechanisms related to the integrin-dependent intestinal homing of T cells, the target of Vedolizumab, could identify new potential (mucosal) biomarkers.[6]

Chronic active inflammation in UC develops from a complex pathogenesis that mainly involves T-lymphocyte infiltration of the gut mucosa.[7] The integrin-dependent T-lymphocyte homing to the gut is controlled by different adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and mucosal vascular addressin cell adhesion molecule-1 (MAdCAM-1).[8] ICAM-1 and VCAM-1 can be found in many organs, while MAdCAM-1 is an adhesion molecule that is predominantly present in the peripheral gut mucosa.[8] The integrin α4β7-expressing T lymphocytes bind to MAdCAM-1 controlling adhesion to the endothelium of postcapillary venules (also called high endothelial venules/HEVs) in the intestine, thereby enabling these effector T lymphocytes to access the gut tissue.[8] Lymphocytes in inflamed colonic mucosa seem to adhere to mucosal HEVs, as well as to peripheral lymph node HEVs via interaction between L-Selectin on naive lymphocytes and peripheral lymph node addressin (PNAd) on endothelial cells.[9] Targeting the homing of T lymphocytes to the gut could lead to reduction of inflammatory infiltration. Vedolizumab serves as an anti-homing integrin by blocking the binding of α4β7 to MAdCAM-1. It is directed against an epitope of the β7 chain, which is only accessible in combination with the α4 chain. Binding of Vedolizumab to α4β7 leads to internalization of the antibody-integrin complex.[10]

To realize an algorithm for positioning biologics, mechanistic-related factors including mucosal response predictors, are of great importance, but currently not available. In this study, we hypothesized that mucosal markers related to integrin-depen-

dent homing at baseline differ between Vedolizumab responders and non-responders and thus can potentially be used as markers predicting treatment response in individual patients. In order to investigate this hypothesis, we prospectively studied the expression of MAdCAM-1, PNAd and α4β7 in pre- and post-treatment colonic biopsies in correlation to clinical and endoscopic response in biological naive UC patients initiating treatment with Vedolizumab.

Patients and methods

Study population

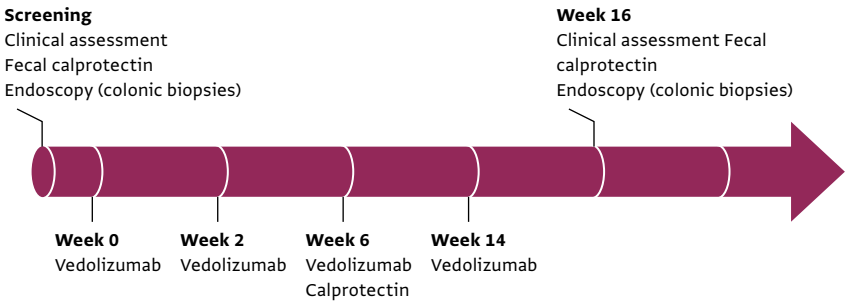
This pilot study was performed at the Department of Gastroenterology and Hepatology in Rijnstate Hospital in Arnhem, a secondary care IBD center in the Netherlands. After written informed consent, we prospectively included adult, biological naive UC patients with a relapse of disease, who were planned to escalate therapy according to the step-up approach to Vedolizumab, without first starting steroids. In addition, we retrospectively included UC patients who were first treated with anti-TNFα before receiving Vedolizumab to compare with biological naïve patients.

Diagnosis of UC was established by fulfilling accepted international clinical and endoscopically diagnostic criteria.[11] Disease phenotype was assessed in line with the Montreal classification.[12] Only UC patients previously treated with 5-aminosalicylates and/or thiopurines were included. Patients who were treated with biologicals at any time before or used steroids within 3 months before inclusion were excluded. Exacerbation of disease was defined as clinical symptoms of diarrhoea, rectal blood loss, abdominal pain or weight loss combined with moderate to severe endoscopic disease according to the Mayo score (Mayo 2-3).

Timeline

The timeline of the study protocol can be found in **Figure 1**. After inclusion patients received Vedolizumab intravenously at weeks 0, 2, 6 and 14, with clinical and endoscopical evaluation at week 16. The Vedolizumab dosage was not adjusted during the study period.

Figure 1 *Timeline for UC patients participating in the study.*



According to most recent literature, clinical response at week 16 was defined as a reduction in the Partial Mayo score with ≥ 2 points, whereas clinical remission was reached when the total Partial Mayo score was 2 or lower. Endoscopic response was defined as a decrease in Mayo score with ≥ 1 grade and Mayo 0 was considered remission. Fecal calprotectin was analysed during screening and at week 16 to confirm clinical and endoscopical response.

Both at screening and evaluation of disease at week 16, eight colonic biopsy specimens were obtained from the macroscopically most inflamed sections. When there was no inflammation present at 16 weeks, biopsy specimens were taken from the same location as during initial endoscopy. Histological activity was evaluated conform regular pathological scoring systems.

Immunohistochemistry

At screening and evaluation (week 16) colonoscopy, two biopsies from inflamed colonic mucosa were incubated in 4% formaldehyde for immunohistochemistry. These biopsies were prepared through dehydration, clearing and infiltration with paraffin in a Leica ASP6025 tissue processor. From formalin-fixed, paraffin-embedded blocks of biopsied specimen of colonic mucosa 3µm thick sections were cut and selected for immunohistochemistry. Immunostaining was performed with hematoxylin and eosin (Klinipath) and the following monoclonal antibodies: CD3 (Novocastra, clone LN10, 1:50, marking T cells), CD20 (Thermo scientific, clone L-26, 1:125, marking B cells), ERG (Ventana, clone EPR3864, demonstrating only nuclei of endothelial cells on all blood vessels), MECA-79 (Santa cruz Biotechnology, clone MECA-79, 1:350, demonstrating 6-sulpho-sialyl Lewis on core-1 branched O-linked sugars (PNAd)), MECA-376 (Hycult Biotech, clone 314G8, 1:50, marking MAdCAM-1). Slides were incubated with these antibodies in an automatic immunostainer (Ventana Benchmark Ultra, Eindhoven, the Netherlands). After performing immunostaining, these slides were scanned with an Intellisite high-resolution scanner (Philips ultra-fast scanner 1.6 RA; Philips Digital Pathology, Best, The Netherlands) and analysed within the IntelliSite Pathology Solution Image Management System (IMS, Philips Digital Pathology, Best, The Netherlands).

Each biopsy specimen was examined with respect to the total surface area of the biopsy (including follicular and extrafollicular tissue) in square millimetres (mm^2), the number of lymphoid follicles and absolute numbers of ERG^+, $PNAd^+$ and $MAdCAM\text{-}1^+$ bloodvenules within the extra- and intrafollicular surface at 40x magnification. The proportion of $PNAd^+$ and $MAdCAM\text{-}1^+$venules among all ERG^+venules was displayed in percentages (% $PNAd^+$ venules/ERG^+venules and % $MAdCAM\text{-}1^+$ venules/ERG^+venules).

Flowcytometry

For the analysis of α4β7 expression on T cells in colonic mucosa, six biopsy specimens were collected in a phosphate-buffered saline solution at 2-8 degrees during screening

and evaluation endoscopy. Flow cytometric analysis (FACSCanto, BD BiosciencesTM) was performed within 8 hours using mechanical preparation of a single cell suspension.

Two hundred µL of the total cell suspension was used and stained with antibodies against CD3 (APC, Becton Dickinson Biosciences USA), CD103/αE (FITC, Becton Dickinson Biosciences USA) and β7 integrin (PerCP-Cy5-5, BioLegend). (**Table 1**) Different CD103 and β7 subpopulations were reported as a percentage of the total $CD3^+$ population. The total $\beta7^+CD3^+$ T-cell population minus the $CD103^+CD3^+$ T-cell population was considered to be representative for the percentage $\alpha4\beta7^+CD3^+$ T cells.

Table 1 *Gating strategy flowcytometry*

FITC	PE	PerCPCy5.5	PE-Cy7	APC	APC-H7
CD103	Ki-67	Beta7	Beta 1	CD45RO	CD3

Statistical analysis

SPSS statistical software (version 25.0.0.0; IBM Corp, Armonk, NY, USA) and GraphPad Prism (Graphpad Software version 7.0, La Jolla, CA, USA) were used to perform statistics. Depending on skewness, the paired T-test or Wilcoxon signed rank test was used to study differences between the expression of PNAd and MAdCAM-1 on colonic bloodvenules at screening and evaluation at week 16. Differences between responders and non-responders during screening were studied using the independent T-test or Mann-Whitney U test. Statistically significant levels are indicated by two-sided p-values of 0.05.

Ethics

The study protocol (NL3633.091.17) was approved by the research ethics committee of the Radboud University Nijmegen Medical Centre (CMO Regio Arnhem-Nijmegen). The procedures were performed in accordance with the Declaration of Helsinki (version 9, 19 October 2013).

Results

Study population

Between 2018 and 2020, 21 UC patients were prospectively included, for baseline characteristics see **Table 2**. Twenty UC patients on 5-ASA and/or thiopurines, had left-sided colitis or pancolitis, one patient (4.8%) had severe, resistant proctitis. The endoscopic severity of disease was moderate in 14 patients (67%) and severe in 7 patients. Five UC patients who were treated with Vedolizumab after they were exposed to anti-TNFα therapy were retrospectively included to compare with biological naïve patients.

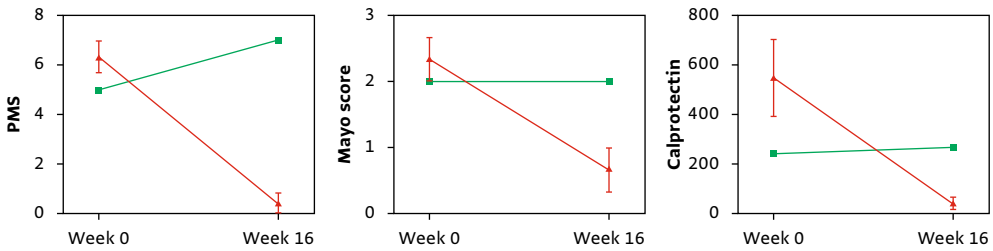
Table 2 *Baseline characteristics.*

	Biological naive UC patients N=21	UC patients priorly treated with anti-TNFα N=5
Gender		
• Female, n (%)	10 (47.6%)	0 (0%)
• Male, n (%)	11 (52.4%)	5 (100%)
Age	39 (34-53)	52 (39-69)
Calprotectin	508 (235-776)	610 (280-842)
Clinical Partial Mayo Score	7 (5-7)	6 (5-7)
UC localization		
• E1: Ulcerative proctitis	1 (4.8%)	0 (0%)
• E2: Left-sided UC	10 (47.6%)	4 (80%)
• E3: Extensive UC	10 (47.6%)	1 (20%)
Mayo Endoscopic score		
• Mayo 2	14 (67%)	2 (40%)
• Mayo 3	7 (33%)	3 (60%)
Treatment received just before initiating Vedolizumab	9 (43%)	0 (0%)
• 5-ASA	0 (0%)	0 (0%)
• Steroids	2 (9%)	0 (0%)
• Thiopurine	10 (48%)	0 (0%)
• 5-ASA^{+} thiopurine	0 (0%)	5 (100%)
• Anti-TNFα		

Values expressed in n (%) or as median with interquartile range; 5-ASA, 5-aminosalicylic acid; UC, ulcerative colitis.

Overall, 15 patients reached clinical remission on Vedolizumab 16 weeks after initiation of treatment and 2 patients had a clinical response. In the same group of patients, endoscopic remission was found in 8 patients and endoscopic response in 9 patients. Four patients had neither clinical nor endoscopic improvement (non-responding group).

Figure 2 *Partial mayo score (PMS), endoscopic mayo score and calprotectin decreasing from week 0 to week 16 in responders to Vedolizumab treatment. ▲ Responders (both clinical and endoscopic remitters and responders, N=17) ■ Non-responders (N=4).*



Clinical and endoscopical remission and response were confirmed by a decrease of calprotectin levels in feces from week 0 to week 16 (**Figure 2**). Since we aimed to find mucosal biomarkers, our focus in the analysis is on endoscopic remission or response.

One out of five patients previously exposed to anti-TNFα had endoscopic response to Vedolizumab, whereas the other four were non-responders.

Follicles, PNAd$^+$ and MAdCAM$^+$ venules in colonic biopsies prior to treatment with Vedolizumab

Remitters, responders, and non-responders demonstrated equal numbers of follicles per mm^2 and equal percentages extrafollicular PNAd$^+$ venules of all ERG$^+$ venules in immunostained biopsy specimens at the start of Vedolizumab therapy (**Table 2**).

The percentages MAdCAM$^+$ venules of all ERG$^+$ venules are similar between remitters and responders (both clinical and endoscopic). When compared to non-responders, these percentages are statistically significant higher in remitters and responders (p=0.03, **Table 3**,

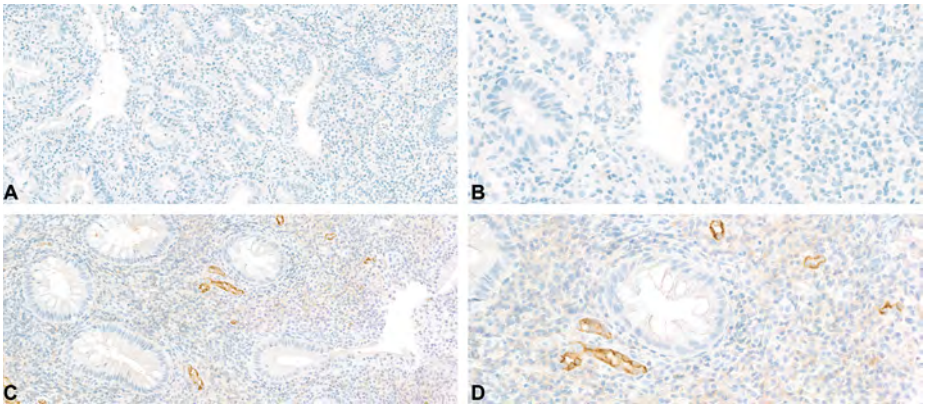
Table 3 *The presence of follicles and (PNAd$^+$, MAdCAM-1$^+$, ERG$^+$) venules in colonic biopsies of biological naive UC patients with active disease before initiating Vedolizumab treatment, comparing patients with no endoscopic response, endoscopic response and endoscopic remission on Vedolizumab using the Kruskal-Wallis test*.*

	Biological naive UC patients N= 21			
	No endoscopic response N= 4	**Endoscopic response N= 9**	**Endoscopic remission N=8**	**P-value***
Total Surface of the Biopsy in mm^2	2.8 (2.0-3.3)	2.1 (1.8-2.6)	2.28 (2.17-2.46)	0.28
Total Follicular Surface in mm^2	0.18 (0.04-0.24)	0.09 (0-0.21)	0.20 (0.03-0.43)	0.37
Follicles				
• Absolute number	5 (0.5-8.0)	2 (0-3)	4 (2-7)	0.30
• Number per mm^2	1.7 (0.27-2.5)	0.93 (0-1.32)	1.49 (0.66-2.56)	0.25
Extrafollicular ERG$^+$ Venules				
• Absolute number	329 (146-487)	297 (159-434)	276 (210-363)	0.87
• Number per mm^2 (density)	115 (59-164)	158 (69-253)	114 (93-148)	0.85
Extrafollicular PNAd$^+$ Venules				
• Absolute number	3.5 (0-39.3)	11 (0-33)	13 (3-34)	0.81
• Number per mm^2 (density)	1.1 (0-12.7)	5.1 (0-17.4)	5.4 (1.1-15.3)	0.65
• As % of ERG$^+$ venules (proportion)	1.1 (0-7.5)	2.4 (0-9.5)	4.2 (1.1-10.8)	0.50
Extrafollicular MAdCAM-1$^+$ Venules				
• Absolute number	2.5 (1.3-6)	23 (19.5-45)	11.5 (8.5-26.3)	*0.01*
• Number per mm^2	1.1 (0.5-2)	11.9 (7.2-20.7)	4.8 (4.1-11.1)	*0.01*
• As % of ERG$^+$ venules (proportion)	1.1 (0.5-2)	10.3 (5.9-10.7)	4.2 (3.0-11.9)	*0.03*

Values expressed in n (%) or as median (interquartile range); ERG, ETS related gene; MAdCAM-1, mucosal vascular addressin cell adhesion molecule-1; PNAd, peripheral node addressin; UC, ulcerative colitis.

Figure 3 for immunohistochemical staining). No statistical differences were found between patients with clinical remission/response and the group with endoscopic remission/response.

Figure 3 *Immunohistochemical staining indicating the presence of MAdCAM-1$^+$ venules in inflamed colonic tissue before treatment with Vedolizumab. Representative photomicrographs of a colonic biopsy of (A) a UC patient with no endoscopic response to Vedolizumab with a magnification of x 20 and (B) with a magnification of x 40, (C) a UC patient with endoscopic response to Vedolizumab with a magnification of x 20 and (D) with a magnification of x 40. MAdCAM-1, mucosal vascular addressin cell adhesion molecule-1; UC, Ulcerative colitis.*



Comparing the biological naïve patients to patients previously treated with biologicals, no differences were found in numbers of follicles or percentages PNAd$^+$ venules (**Table 4**). With regard to percentages MAdCAM$^+$ venules prior to Vedolizumab treatment, higher numbers were found in the responder group priorly treated with anti-TNFα compared to non-responders.

Values expressed in n (%) or as median (interquartile range); ERG, ETS related gene; MAdCAM-1, mucosal vascular addressin cell adhesion molecule-1; PNAd, peripheral node addressin; UC, ulcerative colitis.

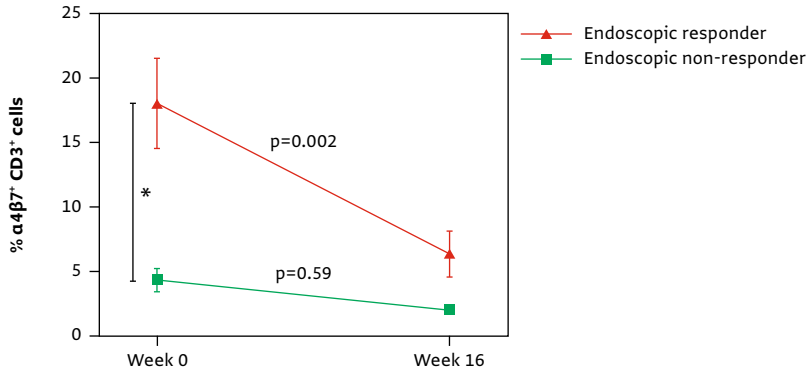
Expression of α4β7 on mucosal T lymphocytes prior to treatment with Vedolizumab

At baseline the median percentage $\alpha4\beta7^+CD3^+$ lymphocytes in remitters and responders (both clinical and endoscopic) was 18% (IQR 12-24) and in non-responders 4% (IQR 3-6) (p=0.002). Comparing baseline to follow up on week 16, a statistical significant decrease of $\alpha4\beta7^+CD3^+$ lymphocytes was demonstrated in remitters and responders (median 8% (3-9), p=0.002), where no difference was seen in non-responders (4%, p=0.59)

Table 4 *The presence of follicles and (PNAd$^+$, MAdCAM-1$^+$, ERG$^+$) venules in colonic biopsies of UC patients prior exposed to anti-TNFα with active disease before initiating Vedolizumab treatment, comparing patients with no endoscopic response, endoscopic response and endoscopic remission on Vedolizumab using the Kruskal-Wallis test*.*

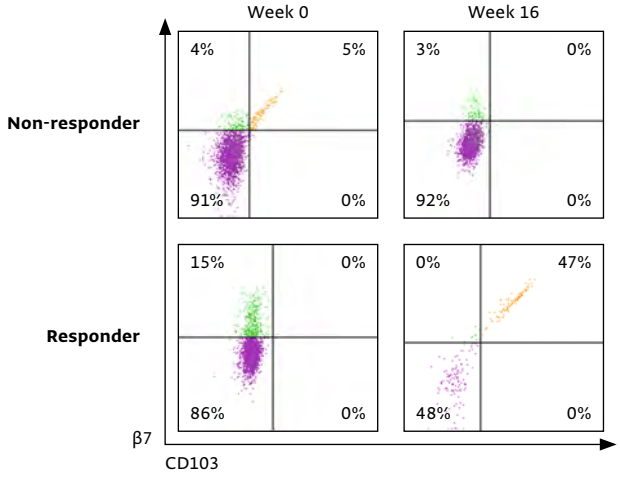
	UC patients prior exposed to anti-TNFα N= 5	
	No endoscopic response N= 4	**Endoscopic response N= 1**
Total Surface of the Biopsy in mm^2	2.62 (1.66-3.37)	2.10
Total Follicular Surface in mm^2	0.02 (0.0-0.18)	0.10
Follicles		
• Absolute number	1.5 (0-3.8)	1
• Number per mm^2	0.51 (0-1.56)	0.48
Extrafollicular ERG$^+$ Venules		
• Absolute number	524 (359-714)	355
• Number per mm^2 (density)	215 (166-259)	169
Extrafollicular PNAd$^+$ Venules		
• Absolute number	21 (6.5-31)	26
• Number per mm^2 (density)	6.8 (2.5-18.3)	12.4
• As % of ERG$^+$ venules (proportion)	3.7 (1.4-7.0)	7.3
Extrafollicular MAdCAM-1$^+$ Venules		
• Absolute number	2.5 (0.25-6.25)	19
• Number per mm^2	0.74 (0.08-2.57)	9.0
• As % of ERG$^+$ venules (proportion)	0.35 (0.04-1.65)	5.35

Figure 4 *Percentages α4β7$^+$ of all CD3$^+$ lymphocytes from flow cytometric analysis in colonic biopsies of UC patients at week 0 and week 16 comparing endoscopic responders with endoscopic non-responders to Vedolizumab. * Significant p-value ≤ 0.01. ▲ Responders (endoscopic remitters and responders , n=17) ■ Non-responders (n=4).*



(**Figure 4**). **Figure 5** displays representative results of flowcytometric analysis of α4β7 within $CD3^+$ T lymphocytes on colonic biopsies. There is no data of the α4β7 expression available in UC patients priorly exposed to anti TNFα, since flowcytometric analysis could not be performed in retrospection.

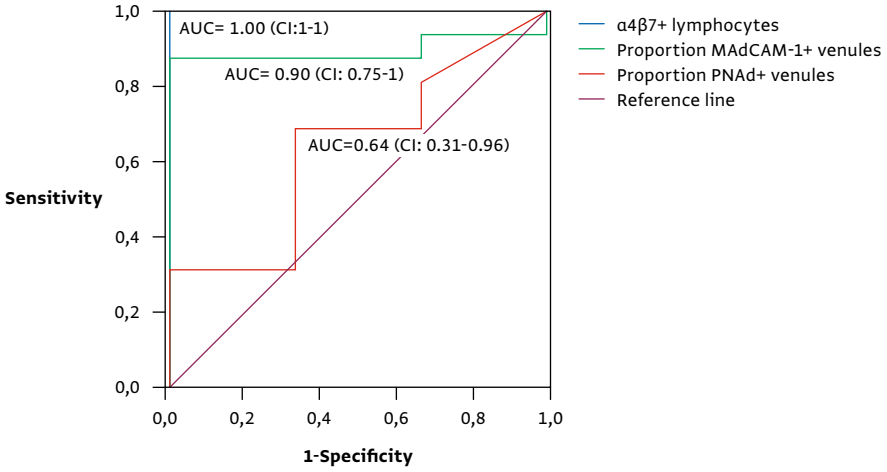
Figure 5 *Representative flowcytometric analysis of α4β7 within $CD3^+$ T lymphocytes on colonic biopsies of an UC patient non-responding to Vedolizumab (top) and an UC patient endoscopically responding to Vedolizumab (bottom) taken during initial endoscopy and at week 16. CD103- and $\beta7^+$ $CD3^+$ lymphocytes are considered to be $\alpha4\beta7^+$.*



Predictive markers for response to Vedolizumab

The potential of the percentage $\alpha4\beta7^+$ $CD3^+$ lymphocytes and the proportion MAdCAM-1^+ and $PNAd^+$ venules to predict response to Vedolizumab was evaluated using receiver operating characteristics (ROC) curves. **Figure 6** shows that the percentage of $\alpha4\beta7^+$ $CD3^+$ lymphocytes provided a complete separation of endoscopic remitters and responders from non-responders (AUC=1, sensitivity 100%, specificity 100%, threshold ≥ 8%).The area under the curve for prediction of endoscopic remission and response versus nonresponse for the proportion MAdCAM-1^+ venules is 0.9 (sensitivity 89%, specificity 100%, threshold ≥ 2.59) and for the proportion $PNAd^+$ venules is 0.6 (sensitivity 61%, specificity 50%, threshold ≥ 2.41).

Figure 6 *Comparing mucosal biomarkers for predicting endoscopic response to Vedolizumab using ROC curves in UC patients. The competency of the percentage α4β7+ lymphocytes and the proportion MAdCAM-1+ and PNAd+ venules to predict response to Vedolizumab assessed by the area under the curve (AUC), specificity and sensitivity displayed next to the reference line.*



Discussion

In this prospective study we demonstrated that percentages of MAdCAM$^+$ of all ERG$^+$ venules and percentages of α4β7$^+$ CD3$^+$ lymphocytes in pre-treatment colonic biopsies are significantly higher in biological naive UC patients responding to Vedolizumab at week 16 compared to non-responders. This difference in % MAdCAM$^+$ venules was also observed in UC patients who earlier failed anti-TNFα therapy.

Previous studies have mainly focused on clinical patient characteristics in order to predict success on treatment with Vedolizumab, since substantial additional effort is required before mucosal biomarker strategies will guide therapeutical decision making in clinical practice. Pre-treatment characteristics, as less severe disease based on clinical acitivity and inflammatory biomarkers,13 no concomitant use of steroids 14 and no prior anti-TNFα treatment,15 have already been described as predictors for achieving early response to Vedolizumab.[5] A response predictive factor during treatment is a higher Vedolizumab concentration in serum after a few weeks of therapy.[16]

However, most clinical characteristics are related to overall disease activity and are not drug specific, which makes them less reliable for decision making in daily practice. In an attempt to identify more specific and objective immunologic biomarkers that predict response to Vedolizumab, integrin-related factors in IBD have been studied. First, it has been demonstrated that integrin α4β7 is differently expressed on T helper cells, and can mainly be found on Th2 and Th17 cells.[6,17] This suggests that selection of patients who might respond to Vedolizumab should rather be based on baseline levels of α4β7 expressing T cells in serum and gut.

Most research on integrin-related biomarkers for Vedolizumab describe associations between changes of serological markers in time and treatment outcomes. In one study, UC patients with better outcomes, showed increasing concentrations of s-α4β7 and decreasing concentrations of s-MAdCAM-1, s-VCAM-1, s-ICAM-1 and s-TNF during treatment with Vedolizumab. No predictive value for efficacy of Vedolizumab was found using pre-treatment levels of these soluble markers.[18] Another study described lower baseline frequencies of peripheral blood $\alpha4\beta7^+$ CD4 T cells in patients with clinical response to Vedolizumab treatment, without a clear explanation by the authors. It concerned a small group of IBD patients previously exposed to anti-TNFα treatment.[19] On the other hand, in another group, higher pre-treatment α4β7 expression on effector (Tem) and terminal effector memory (Temra) T cells in peripheral blood, was demonstrated in anti-TNF experienced IBD patients responding to Vedolizumab.[20] Increased pre-treatment numbers of $\alpha4\beta7^+$ CD4 T cells suggest a more important pro-inflammatory role for the $\alpha4\beta7^+$ dependent homing and therefore it might be more promising to use Vedolizumab in these patients.

Others have studied levels of serum chemokines in a small group (N= 11) of anti-TNFα experienced CD and UC patients at baseline and during Vedolizumab treatment. A suggestion was made that higher baseline serological concentration of CCL13 might predict response to induction therapy (AUC 0.833; 95% CI 0.58 -1.00) but the authors themselves, are cautious in drawing strong conclusions given the small number of patients.[21] CCL13 secretion leads to overexpression of adhesion molecules, such as MAdCAM-1 in endothelial cells and is therefore potentially related to lymphocyte homing.[22]

A retrospective dynamic adhesion assay in simulated intestinal vessels in anti-TNFα exposed UC patients demonstrated that more peripheral blood $CD4^+$ T cells bind to MAdCAM-1 in responders compared to non-responders prior to Vedolizumab treatment.[23] During in vitro treatment, adhesion of $CD4^+$ T cells to MAdCAM-1 was stronger inhibited in responders than in non-responders.[23]

Fewer studies have been performed on mucosal level. A prior study on colonic biopsies, in a small cohort of UC patients (N=7), priorly exposed to anti-TNFα therapy, described higher baseline frequencies of mucosal α4β7 expressing Th1, Th2 and Th17 $CD4^+$ T cells in the lamina propria, in responders to Vedolizumab.[24]

In the present study, pre-treatment percentage of MAdCAM-1$^+$ venules and α4β7$^+$ CD3$^+$ lymphocytes in mucosal biopsies were significantly higher in biological naive UC patients that responded to Vedolizumab. The majority of the anti-TNFα exposed patients included in our study (four out of five patients), displayed low MAdCAM-1 percentages and did not respond to Vedolizumab.

Our results support the hypothesis that, in Vedolizumab responders T-cell homing to the gut mucosa is more driven by binding of α4β7$^+$ CD3$^+$ lymphocytes to MAdCAM-1 and potentially less by interaction of T cells with other adhesion molecules (VCAM-1 and ICAM-1). This assumption was corroborated by findings in 'in vivo' mouse models, where blocking α4β7 homing was compensated by homing of effector T lymphocytes via α4β1/VCAM-1 pathway mainly in ileal Crohn's disease.[25] The use of this alternative pathway may ensure access to the gut in order to maintain or even increase inflammation.[26]

In a recent study, numbers of MAdCAM positive capillaries were lower in CD patients with active disease failing anti-TNFα therapy compared to biological naïve patients. The authors suggest that active inflammation in the anti-TNFα exposed patients may be less dependent on α4β7 mediated homing and therefore may not optimally respond to Vedolizumab treatment.[27] Our study supports the assumption that low baseline MAdCAM expression is predictive for failure of Vedolizumab treatment. A wide range in MAdCAM expression was reported in newly untreated diagnosed UC patients, implying that low MAdCAM levels can not only be clarified by prior anti-TNFα treatment.[28]

The strength of the present study is that we prospectively investigated UC patients with moderate to severe disease activity without concomitant use of corticosteroids or previous exposure to anti-TNFa therapy. Besides, we studied mucosal instead of serological biomarkers, providing a more accurate reflection of the target molecule on the site of inflammation. The relative small sample size of the present cohort might be considered as a limitation, but still we found significant results that support our hypothesis. Methods as immunophenotyping by flowcytometry are challenging to implement in daily practice since biopsies must be investigated within a few hours after withdrawal and are quite expensive techniques. Since immunohistochemistry is more widely used in endoscopic centers and less costly, its clinical utility is superior to flowcytometry. Therefore the measurement of MAdCAM-1 on endothelial venules by immunohistochemistry might improve the feasibility of response predictors for Vedolizumab in the daily practice.

In conclusion, our findings strengthen the approach of assessing mucosal biomarkers such as the frequencies of α4β7$^+$CD3$^+$ lymphocytes and MAdCAM-1$^+$ venules before initiating treatment with Vedolizumab. After validation in a larger patient cohort, implementation of these biomarkers related to integrin-dependent homing in clinical practice might lead to a more target based, personalised and cost-effective therapeutic strategy in UC.

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PART II
RESIDENT T-CELL
POPULATION IN IBD

CHAPTER 4

Intestinal CD103$^+$CD4$^+$ and CD103$^+$CD8$^+$ T-cell subsets in the gut of inflammatory bowel disease patients at diagnosis and during follow-up

INFLAMMATORY BOWEL DISEASE 2019 AUG 20;25(9):1497-1509

Britt Roosenboom

Peter J. Wahab

Carolijn Smids

Marcel J.M. Groenen

Elly van Koolwijk

Ellen G. van Lochem

Carmen S. Horjus Talabur Horje

Abstract

BACKGROUND CD103 is proposed to be a potential therapeutical target in IBD, as it can form a heterodimeric integrin with β7 (Etrolizumab, anti-β7 integrin) on epithelial T cells. Therefore we aimed to study the frequencies of different intestinal $CD103^+$T-cell subsets, both $CD4^+$ and $CD8^+$, in newly diagnosed, untreated IBD patients at baseline and during follow-up, compared to healthy controls.

METHODS Intestinal biopsies from inflamed segments during colonoscopy and peripheral blood samples were prospectively taken from IBD patients at diagnosis and during follow-up. Blood and single cell suspensions from biopsies were analyzed for $CD103^+$ T-cell subpopulations by flow cytometry and expressed as median percentages of the total T-cell population.

RESULTS In total, 75 Crohn's disease, 49 ulcerative colitis patients and 16 healthy controls were included. At presentation, IBD patients displayed lower percentages of $CD103^+$T-cell subsets in inflamed biopsies: 3% (1-5) $CD103^+CD4^+$ in IBD, versus 5% (5-7) in healthy controls (p=0.007) and 9% (4-15) $CD103^+CD8^+$ compared to 42% (23-57) in healthy controls (p=0.001). The majority of intestinal T cells was composed of $CD103^-CD4^+$ T cells (65% (52-74)) in IBD compared to 30% (21-50) in healthy controls (p=0.001). In patients with endoscopic remission during follow up (n=27), frequencies of $CD103^+$ and $CD103^-$T-cell subsets were comparable to healthy controls.

CONCLUSION At diagnosis, active inflammation in IBD was associated with decreased percentages of both $CD103^+CD4^+$ and $CD103^+CD8^+$T-cell subsets in colon and ileum biopsies. In active disease during follow up these T-cell populations remained low but increased in remission to values comparable with healthy controls. A shift towards more $CD103^-$T cells was observed during active inflammation.

Introduction

Aberrant and inordinate immune responses to environmental triggers may cause chronic active mucosal inflammation in a genetically susceptible host, leading to chronic gut inflammation disorders such as Crohn's disease (CD) and ulcerative colitis (UC). T lymphocytes are pivotal in the immune response associated with inflammatory bowel disease (IBD).[1,2]

Regularly, the gut homing phenotype, characterized by expression of the α4β7 integrin, is induced on naïve T cells after encounter with their cognate antigen in the Gut-Associated-Lymphoid-Tissue (GALT), smaller lymph aggregates and mesenteric lymph nodes.[3] In IBD, increased recruitment of these primed effector T cells to the intestinal mucosa has been described, by the binding of the α4β7 integrin to MAdCAM-1 on endothelial cells.[3,4] After entrance to the lamina propria, in the presence of TGF-beta, $\alpha E\beta7^+$ may be brought to expression on specific T-cell subsets.[5,6]

The $\alpha E\beta7^+$ integrin is considered to be of importance in modulating homeostasis of memory T cells by mediating selective retention of these T lymphocytes in the intestinal epithelia and lamina propria through its binding to E-cadherin.[7] αE can only form a heterodimer with β7, while β7 can bind to both αE and α4. Gene expression of $\alpha E\beta7^+$ differs from the expression of this integrin on the cell surface, because intracellular regulatory events might inhibit translation of mRNA to $\alpha E\beta7^+$ integrin present on the cell surface.[8] $\alpha E\beta7^+$ integrin can be identified with monoclonal antibodies recognizing the CD103 subunit on the cell surface by flow cytometry, therefore we refer to $CD103^+$ when αE is expressed on T cells.[9]

Currently, treatment options preventing the gut-specific migration of T cells in IBD, are being extended with antibodies against different integrins, like α4β7 (anti-α4β7, Vedolizumab) and $CD103^+(\alpha E)\beta7$ (anti-β7, Etrolizumab) and adhesion molecules, such as MAdCAM-1 (anti-MAdCAM-1, PF-00547659).[3,10] In animal models, blocking $\beta7^+$ by a combination of anti-α4β7 and anti-$\alpha E\beta7$ antibodies, suppressed accumulation of the $CD103^+CD8^+$ T cells but had no effect on the accumulation of the pro-inflammatory $CD103^+CD4^+$ T cells in the gut mucosa.[11] Assessment of the relative numbers of different $CD103^+(\alpha E)\beta7^+$ T-cell subsets in the inflamed gut mucosa could be useful in order to identify patients suitable for different treatment strategies.

The major T-cell subsets, including $CD4^+$ and $CD8^+$ T cells, may have different functions within the mucosal $CD103^+$ T-cell population. A potential pro-inflammatory role was allocated exclusively for the $CD103^+CD4^+$ subset while functional investigation could not confirm this role for the $CD103^+CD8^+$ T cells in the gut of UC patients.[12] The latter seems to belong to the tissue-resident-memory T cells (TRMs) with an immunosurveillance and protective function in different human tissues, also presumable for the intestinal mucosa.[13] CD69 is another marker for TRMs and distinguishes the resident population from circulating memory T cells together with CD103. CD69 is also well

known as a marker of activated cells, most often lymphocytes and natural killer cells.[14] However, the frequencies of the different $CD103^+(CD69^+)CD4^+$ and $CD103^+(CD69^+)CD8^+$ T-cell subsets have not been studied in newly diagnosed IBD patients, so far.

In our previous study, we observed lower percentages of overall $CD103^+$ T cells in IBD patients with active disease compared to healthy controls in biopsies of ileal and colonic mucosa analysed together.[15] The present study is a continuation of this work, expanding the patient population, analysing ileal and colonic biopsies separately for the determination of the percentages of $CD103^+CD4^+$ and $CD103^+CD8^+$ subsets, performing additional phenotypical analysis and studying both newly diagnosed untreated IBD patients at presentation and during follow-up.

Patients and methods

Patients

IBD patients were prospectively recruited at the Department of Gastroenterology and Hepatology in Rijnstate Hospital in Arnhem, The Netherlands, a referral hospital for IBD patients. Patients suspected of IBD based on clinical symptoms (chronic diarrhoea, rectal blood loss, abdominal pain or weight loss) and/or calprotectin values, underwent ileocolonoscopy as part of their initial diagnostic work-up. The diagnosis of IBD was based on clinical, endoscopic, and histopathological features according to the ECCO guidelines.[16,17] Exclusion criteria consisted of concomitant or previous use of immunosuppressive medication, the presence of other autoimmune disorders or malignancies. We included healthy controls (HC) that underwent ileocolonoscopy for polyp surveillance or iron deficiency. Enrolled HC had no history of autoimmune diseases. During ileocolonoscopy macroscopically normal colonic and ileal mucosa of HCs was identified and subsequently microscopically confirmed. At the time of inclusion, all IBD patients were naïve to immunosuppressive treatment (e.g. steroids, thiopurines, aminosalicylates and biologics). Follow up ileocolonoscopy was performed as part of standard care in 42.7% of the included IBD patients.

After initial diagnosis of IBD, treatment was initiated according to the step-up approach. CD patients started with corticosteroids, followed by maintenance treatment with immunomodulators (primarily thiopurines) and when ineffective anti-TNFα treatment was prescribed. Preparations with aminosalicylates or corticosteroids were the first options of treatment in patients with UC. In case of non-response, thiopurines or anti-TNFα treatment was initiated. The follow-up period ranged from diagnosis until the last visit at the outpatient clinic. We reported the highest treatment step that was taken in order to reach remission in this follow-up period. This step was defined as the last treatment applied using the step up approach.

Definition of disease activity

The Mayo score (0-3 scale) was used to assess endoscopic severity of disease activity at primary diagnosis and during follow up endoscopy in UC patients. Patients with inactive disease were in endoscopic remission, defined as a Mayo score of 0.18 The simple endoscopic score for CD patients (SES-CD, scale from <4 to >19) was used to assess endoscopic severity of disease activity at primary diagnosis and during follow up in CD patients. Patients with inactive disease were in endoscopic remission, defined as a SES-CD <7 and <4 for ileum-only CD patients.[19]

Location and behaviour of disease was reported for both entities according to the Montreal classification.[18] The Harvey-Bradshaw index was reported for evaluation of clinical complaints in Crohn's disease and Mayo severity score for UC patients.[20]

Methods

Tissue handling and flow cytometry

At the time of initial diagnosis and during follow-up ileocoloscopy 4-6 biopsies were taken from the most inflamed ileal and/or colonic parts of the mucosa, respectively. When ulcerations were present, these biopsy specimens were taken from the inflamed mucosa on the edge of the ulcerations.

In case of remission during follow-up endoscopy, biopsied specimens were taken from the same segments as at initial investigation. If patients had active disease during follow-up endoscopy, biopsies were taken from the most inflamed mucosal areas. Biopsied specimens from healthy controls were taken from ileum and colon in order to perform flow cytometric analysis and regular histological evaluation. Parallel to the flow cytometric analysis, biopsies were histologically evaluated for the confirmation of chronic active inflammation. Specimens for flow cytometric analysis were collected in a phosphate-buffered saline solution at 2-8 degrees and analysed within 8 hours. In order to preserve all cell surface proteins for phenotyping we avoided enzymatic digestion and used mechanical preparation of a single cell suspension. Hereto, specimens were pooled and blended in Hanks'/1% bovine serum albumin using a 70-mm gaze and spatula followed by Ficoll density gradient centrifugation.The homogenate was resuspended, after washing, in 0,5 mL Hanks'/1% bovine serum albumin. The concentration of mononuclear cells in the suspension was estimated by microscopic counting with a KOVA glasstic slide (Hycor Biomedical Ltd., Penicuik, United Kingdom) following the same protocol as in our previous study.[15]

Two hundred µL of the total cell suspension was used for flow cytometric analysis (FACSCanto, BD BiosciencesTM). The total intestinal $CD3^+$ T-cell population was reported as percentage from the whole lymphocyte population. The different $CD3^+$ T-cell subpopulations: $CD8^+$, $CD4^+$, $CD103^+$, $CD103^-$ $CD69^+$, CD69-, $FoxP3^+$, FoxP3- and Ki-67^+ T cells were reported as a percentage of the total $CD3^+$-population. $CD103^+(CD69^+)$ and $CD103^-(CD69^+)$ T cells were also expressed as a percentage of the $CD3^+CD4^+$ and the

$CD3^+CD8^+$ subset, respectively. Antibodies and reagens used for flow cytometry were all obtained from Becton Dickinson Biosciences USA (**Supplementary Table 1**).

Peripheral blood handling and flow cytometry

Peripheral blood was withdrawn after initial ileocolonoscopy for measurement of C-reactive protein (CRP) and for immunophenotyping. Immunophenotyping was performed on whole blood (100 µl per monoclonal combination) followed by erythrocyte lysing using FACS lysing solution (BDBiosciences) and permeabilization (in case intracellular staining: FACS Fix/Perm solution BD Biosciences).

Statistical analysis

The Shapiro-Wilk test was used to test whether the sample was distributed normally. Categorical characteristics of patients were presented as a number (n) with percentage and analysed using the Chi-square test in case of normal distribution, otherwise, the Fisher's exact test was performed. Medians of continuous variables were reported with 25th and 75th percentile (interquartile rang/IQR) in case the distribution was not normal. In case of a normal distribution, different T cell subsets were analysed with the independent T-test, otherwise, the Mann-Whitney U test was performed. Patients in the follow up group were compared with their own baseline values using the Wilcoxon signed ranks test or the paired T-test. The Spearman test was used to test the correlation between the different T-cell subsets and the SES-CD score in CD and the Mayo in UC patient, if both variables were continuous,we performed the Pearson rank test. Statistical significance was accepted if the probability of a type I error did not exceed 5%. Data were analysed with SPSS statistics (version 22.0.0.0; IBM Corp, Armonk, NY, USA) and GraphPad Prism (GraphPad Software version 7.0, La Jolla, CA, USA).

Ethics

The study protocol (NL28761.091.09) was approved by the research ethics committee of the Radboud University Nijmegen Medical Centre (CMO Regio Arnhem-Nijmegen). Written informed consent was obtained from each participating patient before any study-related procedure was performed. The procedures were performed in accordance with the Declaration of Helsinki (version 9, 19 October 2013).

Results

Study population

The baseline characteristics of all patients and HC are presented in **Table 1**.In total 75 CD patients, 49 UC patients and 16 HC were included. CD and UC groups were comparable for age and gender (p=0.37 and p=0.15). CD patients had higher baseline CRP levels

(p=0.001), more extra-intestinal manifestations (p=0.004) and fewer family members with IBD (p=0.005) compared to UC patients. More CD patients were smokers at initial presentation compared to UC patients (p=0.004). CD patients also had a longer history of complaints before diagnosis during initial ileocolonoscopy (p=0.017).

Table 1 *Patient demographics. CD, Crohn's disease, UC, Ulcerative Colitis, IQR, Interquartile range.* Significant p-value ≤0.05.*

	CD (n=75)	UC (n=49)	HC (n=16)	p-value CD-UC
Median age at diagnosis in years (IQR)	(21-40)	30 (26-40)	43 (33-55)	0.37
Gender				
• Female, n (%)	49 (65.3%)	30 (61.2%)	14 (87.5%)	0.15
• Male, n (%)	26 (34.7%)	19 (38.8%)	2 (12.5%)	
Smoking at diagnosis				
• Yes	32 (42.7%)	7 (14.3%)	6 (37.5%)	0.004*
• No	43 (57.3%)	41 (83.7%)	10 (62.5%)	
Extra-intestinal manifestations				
• Yes	18 (24.0%)	2 (4.1%)	0 (0%)	0.004*
IBD in family				
• Yes	7 (9.3%)	15 (30.6%)	1 (6.3%)	0.005*
Duration of complaints before diagnosis				0.017*
• <3 months	27 (36.0%)	21 (42.9%)	-	
• 3-6 months	15 (20.0%)	18 (36.7%)		
• > 6 months	33 (44.0%)	9 (18.4%)		
CRP at initial diagnosis	21 (9-61)	3 (1-14)	1 (1-6)	0.001*
Median clinical follow-up period in months (IQR)	32 (19-70)	(9-41)	-	0.601
Baseline Mayo endoscopic score, n (%)			-	-
• Mayo 0	-	0 (0%)		
• Mayo 1		9 (18.4%)		
• Mayo 2		30 (61.2%)		
• Mayo 3		10 (20.4%)		
Baseline Montreal UC, n (%)				
• Extent	-	11 (22.4%)	-	-
○ E1: Ulcerative proctitis		17 (34.7%)		
○ E2: Left-sided UC		21 (42.9%)		
○ E3: Extensive UC		0 (0%)		
• Severity		14 (28.6%)		
○ S0: Clinical remission		23 (46.9%)		
○ S1: Mild UC		12 (24.5%)		
○ S2: Moderate UC				
○ S3: Severe UC				

HBI score, n (%)				
• <5, remission	3 (4.0%)	-	-	-
• 5-7 mild disease	31 (41.3%)			
• 8-16 moderate disease	33 (44.0%)			
• >16 severe disease	8 (10.7%)			
Baseline SES-CD, n (%)				
• 0-3 inactive disease	0 (0%)	-	-	-
• 4-10 mild disease	26 (34.7%)			
• 11-19 moderate disease	33 (44.0%)			
• >19 severe disease	16 (21.3%)			
Baseline Montreal CD, n (%)				
• Location	26 (34.7%)	-	-	-
○ L1: ileal	32 (42.7%)			
○ L2: colonic	17 (22.6%)			
○ L3: ilealcolonic	58 (77.3%)			
• Behaviour	13 (17.3%)			
○ B1: non-stricturing, non-penetrating	4 (5.3%)			
○ B2: structuring	11 (14.7%)			
○ B3: penetrating				
○ P: perianal disease				
Highest step in treatment to reach remission				
• 5-ASA	4 (5.3%)	30 (61.2%)	-	0.001*
• Oral steroids	6 (8%)	4 (8.2%)		
• Immunomodulators	41 (54.7%)	12 (24.5%)		
• Anti-TNF	20 (26.7%)	3 (6.1%)		
• MTX	3 (4%)	0 (0%)		
• Anti-integrins	0 (0%)	0 (0%)		
• Resective surgery	1 (1.3%)	0 (0%)		
Follow-up Mayo endoscopic score n=20, n (%)				
[Baseline Mayo score of the follow-up group, n [%]]	-	7 (35%)		
• Mayo 0		[0[0%]]		
• Mayo 1		5 (25%)		
• Mayo 2		[4[20%]]		
• Mayo 3		4 (20%)		
		[10[50%]]		
		4 (20%)		
		[6[30%]]		
Follow-up SES-CD n=32, n (%)				
• 0-3 inactive disease	21(65.6%)	-		
• 4-10 mild disease	7 (21.9%)			
• 11-19 moderate disease	3 (9.4%)			
• >19 severe disease	1 (3.1%)			

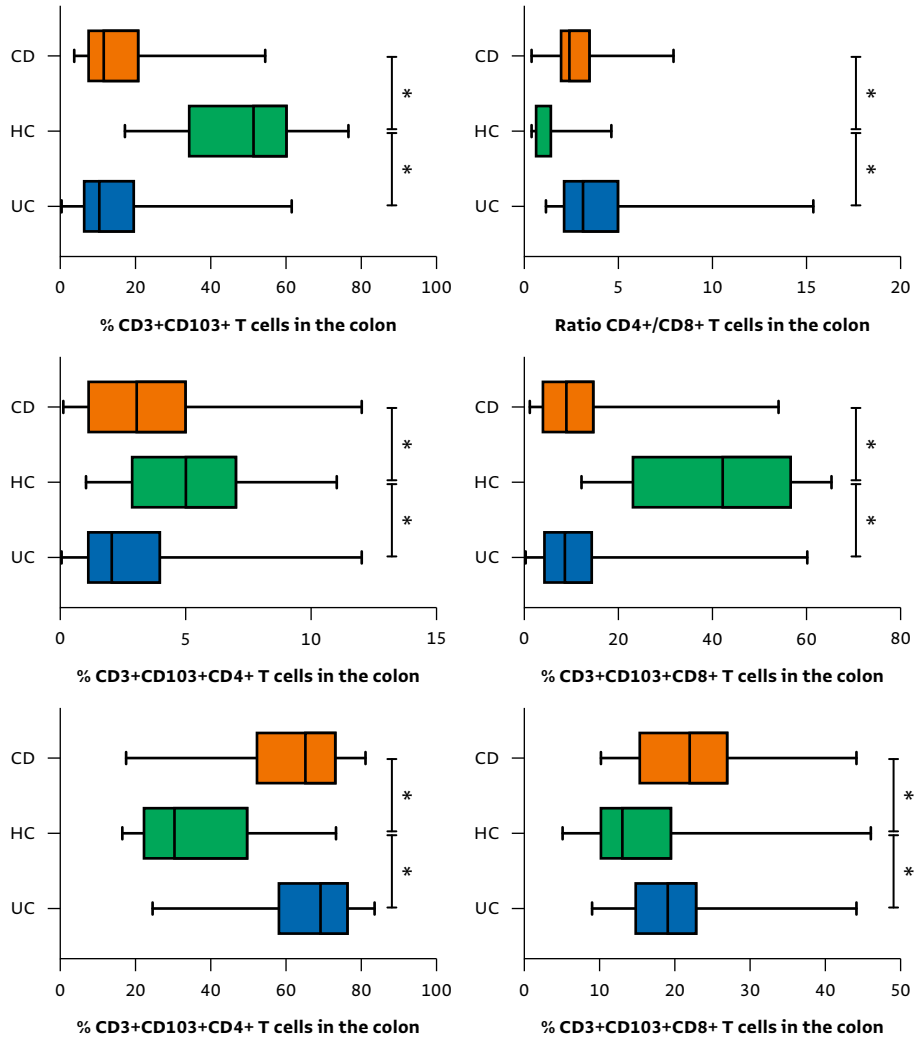
After diagnosis the majority of CD patients needed immunomodulators (n=41 (54.7%)). The majority of UC patients reached remission on aminosalicylate preparations (n=30 (61.2%)). No patients were treated with Vedolizumab or Etrolizumab.

Frequencies of intestinal CD103$^+$T-cell subsets at baseline

Ulcerative colitis

The baseline frequencies of the different intestinal T-cell subsets in UC can be found in **Figure 1** and **Supplementary Table 2**.

Figure 1 *Baseline percentages of CD103$^+$, CD103$^+$CD4$^+$, CD103$^+$CD8$^+$, CD103-CD4$^+$ and CD103-CD8$^+$ within CD3$^+$ T lymphocytes and the ratio CD4$^+$/CD8$^+$ T lymphocytes explored with FACS analysis on colonic biopsies of UC and CD patients with active colon disease compared to healthy controls. * Significant p-value. UC, Ulcerative colitis; CD, Crohn's disease.*



In colonic biopsies of UC patients, lower percentages of $CD103^+$ T cells (11% (6-20)) were found compared to colonic biopsies of HC (52% (34-61), p=0.001). Both $CD103^+CD4^+$ and $CD103^+CD8^+$ T-cell subsets were present in lower percentages (respectively 3%(1-4)) and 9% (5-14)) in UC compared to HCs (respectively 5%(5-7), p=0.002 and 42% (23-57), p=0.001). The $CD103^-CD4^+$ T-cell subpopulation predominates (69%) in colonic biopsies from UC patients at diagnosis compared to HCs, in concordance with a higher $CD4^+$/$CD8^+$ ratio of 3.0 (2.0-4.2) in patients versus 0.7 in HCs (0.6-1.5, p=0.001).

There is no correlation between the severity of disease (mayo score) at diagnosis and the numbers of the different T-cell subsets (**Supplementary Figure 1**).

Crohn's disease

The baseline frequencies of the different colon and ileum T-cell subsets in CD can be found in **Figure 1**, **2** and **Supplementary Table 2**.

Colonic biopsies of active CD patients showed lower percentages of $CD3^+CD103^+$ T cells (11%(7-21)) compared to colonic biopsies of HCs (52% (34-61), p=0.001). Numbers of both $CD103^+CD4^+$ and $CD103^+CD8^+$ T cells were decreased compared to HC (42% (23-57)), with a more pronounced decrease in the $CD103^+CD8^+$ subset (9% (4-15), p=0.001). In line with these results, the majority of the colonic $CD8^+$ T cells (86% (66-100)) and $CD4^+$ T cells (92% (85-97)) were $CD103^-$. The $CD4^+$/$CD8^+$ ratio within the total T-cell population in the colon of CD patients was 2.4 (1.9-3.5) versus 0.7 in colon biopsies taken from healthy controls (0.6-1.5, p=0.001).

There were no statistical differences for any of the analysed subsets between ileal and colonic location in CD patients, nor between colonic CD and UC.

Concomitant peri-anal disease at initial diagnosis of CD was associated with lower percentages of $CD103^+$ T cells, both $CD103^+CD4^+$ and $CD103^+CD8^+$ T-cell subsets, in ileal biopsies (respectively, 5.5% (2.75-8.25), 1.5% (1-2) and 4.25% (2.25-6.5)) compared to active ileal CD without peri-anal activity (respectively 15.0% (7-35) p=0.018, 4.0% (2-10) p=0.039 and 11.0% (6-24) p=0.027). However, there was no significant correlation between the SES-CD score and the numbers of the different T-cell subsets (**Supplementary Figure 1**).

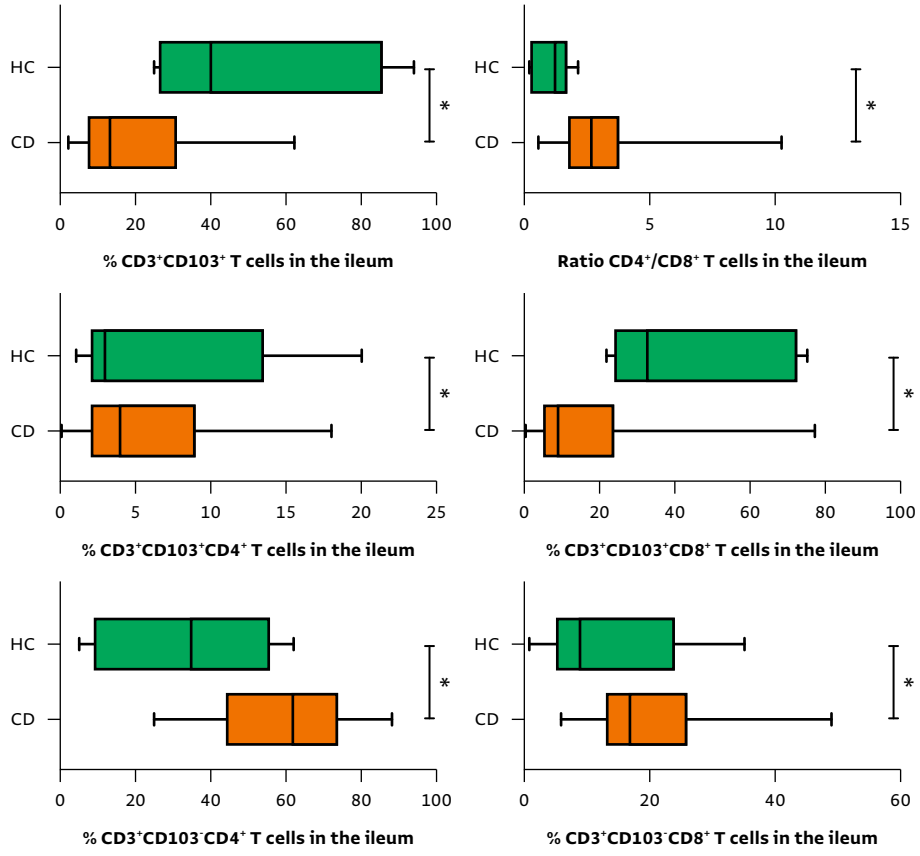
Smoking in CD was associated with higher $CD103^-CD8^+$ T cells (25.0% (17-31)) in the colon biopsies and higher $CD103^+CD4^+$ T cells (6.0% (3-13)) in ileal biopsies compared to non-smoking CD patients (respectively 19.5% (14-24) p=0.011 and 3.0% (1.0-5.75) p=0.03).

Additional phenotypical analysis of the $CD103^+$ T-cell subsets at baseline

CD69 as a marker of cell activation

In a smaller cohort of newly diagnosed IBD patients (UC n=10, CD n=15 and HC n=4) CD69 staining was performed. In the colonic biopsies of UC and CD patients, lower percentages of $CD103^+CD8^+CD69^+$ T cells (7% (5-12) resp. 6% (4-10)) were demonstrated compared to HC (14% (8-21), UC vs HC p= 0.14, CD vs HC p=0.05), similar to lower per-

Figure 2 *Baseline percentages of CD103$^+$, CD103$^+$CD4$^+$, CD103$^+$CD8$^+$, CD103-CD4$^+$ and CD103-CD8$^+$ within CD3$^+$ T-lymphocytes and the ratio CD4$^+$/CD8$^+$ T-lymphocytes explored with FACS analysis on ileal biopsies of CD patients with active ileal disease compared to healthy controls. * Significant p-value. CD, Crohn's disease.*



centages of CD103$^+$CD4$^+$CD69$^+$ T cells (UC 3% (2-6), CD 2% (1-4), HC 5% (3-9), UC vs HC p=0.37, CD vs HC p=0.11) (**Table 2**).

Overall percentages of CD103$^+$CD69$^+$ T-cell subsets were higher in the ileum of CD patients and HC compared to percentages in their colonic mucosa. Ileal CD was associated with statistic significant lower percentages of CD103$^+$CD69$^+$ (both CD8$^+$ (19% (2-28)) and CD4$^+$ (5% (2-8))) T cells compared to the ileum of HC (resp. 40% (29-61), p=0.05 and 14% (9-21), p=0.01) (**Table 2**).

Table 2 *Baseline percentages of CD103$^+$CD4$^+$CD69$^+$, CD103$^+$CD8$^+$CD69$^+$, CD103-CD4$^+$CD69$^+$, and CD103-CD8$^+$CD69$^+$ within CD3$^+$ T lymphocytes explored with FACS analysis in Crohn's disease, ulcerative colitis and healthy controls. Median percentages (IQR). CD, Crohn's disease, UC, Ulcerative Colitis, IQR, Interquartile range. * Significant p-value ≤0.05. N= amount of analyzed biopsies.*

	CD		UC	HC		P-value
Location of biopsies inflamed mucosa at first presentation	**Colon (n=10)**	**Ileum (n=10)**	**Colon (n=10)**	**Colon (n=4)**	**Ileum (n=4)**	
CD3$^+$CD103$^+$CD8$^+$CD69$^+$%†	6.42 (4.3-10.4)	18.7 (1.5-27.8)	7.5 (4.6-11.8)	14.1 (8.3-21.2)	39.7 (29.1-60.6)	10.14 20.05* 30.05*
CD3$^+$CD103-CD8$^+$CD69$^+$%†	4.4 (3.3-7.2)	2.4 (1.7-5.7)	5.7 (4.2-6.4)	3.1 (1.7-3.7)	1.6 (1.4-1.9)	10.01* 20.05* 30.08
CD3$^+$CD103$^+$CD8$^+$CD69-%†	0.6 (0.4-0.8)	1.1 (0.4-1.6)	0.8 (0.4-1.8)	0.8 (0.6-0.9)	0.5 (0.1-0.9)	11.01 20.24 30.11
CD3$^+$CD103-CD8$^+$CD69-%†	11.9 (7.9-12.7)	6.2 (2.1-9.4)	6.0 (3.5-9.0)	4.6 (3.9-6.9)	1.2 (0.6-5.6)	10.73 20.01* 30.11
CD3$^+$CD103$^+$CD4$^+$CD69$^+$%†	2.4 (1.3-4.0)	5.3 (1.8-7.7)	2.7 (1.8-6.1)	5.1 (2.6-9.0)	13.9 (8.8-20.9)	10.37 20.11 30.01*
CD3$^+$CD103$^-$CD4$^+$CD69$^+$%†	32.0 (27.0-38.5)	32.7 (19.2-37.9)	50.4 (34.3-58.1)	45.7 (33.3-47.8)	18.4 (12.3-32.2)	10.37 20.14 30.19
CD3$^+$CD103$^+$CD4$^+$CD69-%†	0.0 (0.0-0.7)	0.4 (0.0-0.7)	0.7 (0.0-0.8)	0.0 (0.0-0.5)	0.4 (0.1-0.6)	10.24 20.84 31.01
CD3$^+$CD103-CD4$^+$CD69-%†	39.9 (32.5-45.2)	33.3 (14.1-50.0)	23.2 (18.2-25.4)	32.1 (20.9-33.3)	17.5 (7.7-23.7)	10.24 20.08 30.11

1 P-value comparison active UC colon with HC colon.
2 P-value comparison active CD colon with HC colon.
3 P-value comparison active CD ileum with HC ileum.
† Percentages within CD3$^+$ T cells

FoxP3 as a marker for regulatory CD4$^+$ T cells

We found low percentages of CD103$^+$FoxP3$^+$CD25$^+$CD4$^+$ T cells both in colon and ileum, ranging from 0-2% of the total lymphocyte population (with no differences between HC and IBD patients, p=0.98). The majority of FoxP3$^+$CD25$^+$CD4$^+$ Tregs turned out to be CD103$^-$.

Ki-67 as a marker for cell proliferation

CD103$^+$ T-cell subsets had a two fold higher fluorescence intensity of Ki-67 compared to CD103$^-$ T-cell subsets (**Table 3**). This suggests a higher proliferation rate of the CD103$^+$ T cells compared to the CD103$^-$ T cells.

Table 3 *Ki-67 expression on CD8$^+$CD103$^+$, CD8$^+$CD103-, CD4$^+$CD103$^+$ and CD4$^+$CD103$^-$ T cells (Geometric mean fluorescence intensity). UC, ulcerative colitis, CD, Crohn's disease, IQR, Interquartile range.*

Location of biopsies inflamed mucosa at first presentation	UC colon (n=11)	CD colon (n=4)	CD ileum (n=4)
CD3$^+$CD8$^+$CD103$^+$ki67	848 (709-1263)	804 (573-1229)	624 (530-727)
CD3$^+$CD4$^+$CD103$^+$ki67	475 (334-894)	510 (384-847)	472 (357-704)
CD3$^+$CD8$^+$CD103$^-$ki67	539 (454-666)	453 (443-480)	594 (445-699)
CD3$^+$CD4$^+$CD103$^-$ki67	226 (221-309)	275 (264-388)	317 (240-434)

CD103$^+$ T-cell subsets in peripheral blood at baseline

Percentages of the CD103$^+$ T-cell subsets in PBMC were very low (<1% within the CD3$^+$ population), with no differences between IBD patients and HC. (**Supplementary Table 3**)

Frequencies of intestinal CD103$^+$T-cell subsets during follow-up

Ulcerative colitis

The frequencies of different intestinal T-cell subsets in UC during follow-up can be found in **Supplementary Table 4**.

Percentages of different intestinal T-cell subsets from UC patients in remission during follow-up were compared with their own baseline and with HC. In these patients, the percentages shifted to levels comparable to HC (**Figure 3,4,5** and **Supplementary Table 4**). There was a statistically significant increase of % CD103$^+$ T cells (both CD4$^+$ and CD8$^+$) in patients in remission during follow-up when compared to their own baseline values. The CD4$^+$/CD8$^+$ ratio in remission (0.7 (0.57-1.53)) was comparable to the ratio in healthy controls (0.7 (0.6-1.5)). In UC patients with active disease during follow-up, T-cell subpopulations were comparable to their baseline levels.

Patients with UC with active endoscopic disease during follow-up had statistically significant lower percentages of CD103$^+$ (both CD4$^+$ (3.0% (1-4)) and CD8$^+$ (6% (2-13))) compared to patients with inactive endoscopic disease (5.0% (3-8) and 38% (18-45), p=0.002). In active disease during follow-up, the CD103$^-$ CD4$^+$ T-cell subpopulation is predominant (76.0% (69-86)).

Crohn's disease

The frequencies of different intestinal T-cell subsets in CD during follow-up can be found in **Supplementary Table 5**.

Percentages of different intestinal T-cell subsets in colonic and ileal tissue from CD patients in remission were compared with their own baseline levels and HC. In the ileal tissue of these CD patients, the percentages of the different intestinal subsets reached levels comparable to HC (**Figure 3,4,5** and **Supplementary Table 5**). This was a statistically significant increase of $CD103^+$ T cells, both $CD4^+$ and $CD8^+$, in the ileum of CD patients with inactive disease during follow-up (p=0.001, p=0.003 and p=0.002). In line with these findings, the percentages of $CD103^-$ T-cell subsets were decreased. The $CD4^+/CD8^+$ ratio in the ileum of patients with inactive disease was 0.73 (0.46-1.15), comparable to the ratio in healthy controls (0.7 (0.6-1.5)).

Figure 3 *Representative flow cytometric analyses of $CD103^+CD4^+$, $CD103^+CD8^+$, $CD103^-CD4^+$ and $CD103^-CD8^+$ within $CD3^+$ T-lymphocytes on A) colonic biopsies of a CD patient with moderate to severe colonic disease during baseline and follow-up endoscopy B) colonic biopsies of a CD patient with moderate to severe colonic disease during baseline endoscopy and inactive disease during follow-up endoscopy C) colonic biopsies of a healthy control.*

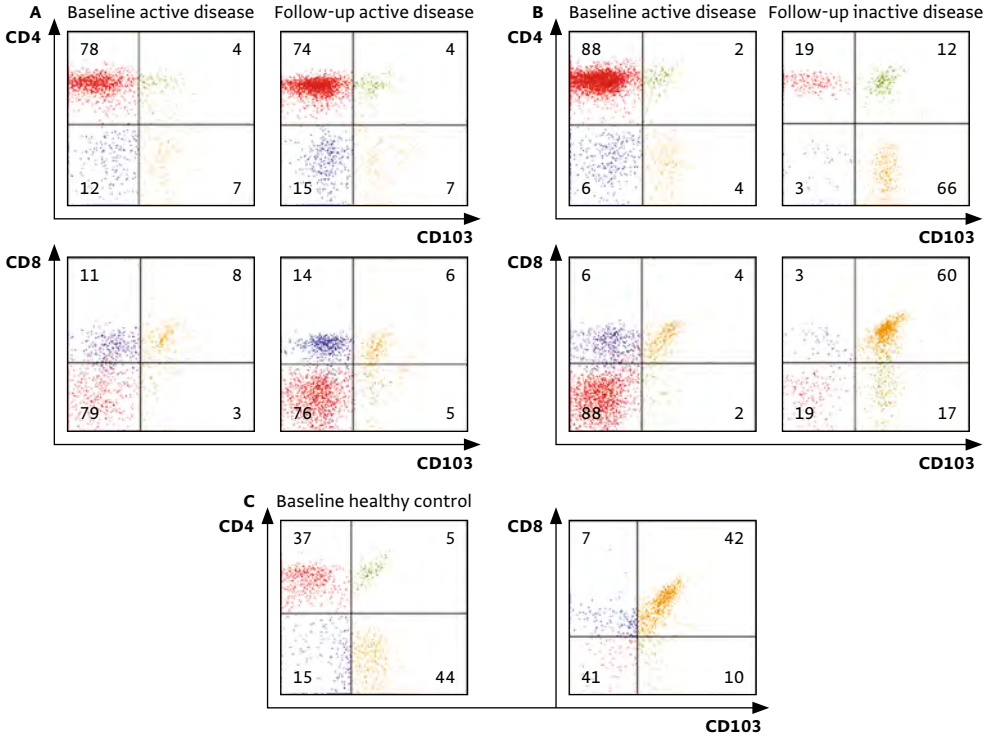


Figure 4 *Intra-individual fold change, expressing the ratio between follow-up and baseline percentages of the different intestinal lymphocyte subsets with either active or inactive endoscopic disease(A:CD3, CD4, CD8; B:CD103$^+$, CD103$^+$CD4$^+$, CD103$^+$CD8$^+$; C:CD103$^-$CD4$^+$, CD103-CD8$^+$). ● Active UC (n= 13) ○ Inactive UC (n= 7) ▲ Active colon CD (n=9) Δ Inactive colon CD (n=10) ■ Active ileum CD (n=6) □ Inactive ileum CD (n=17) * Significant p-value ≤0.05.*

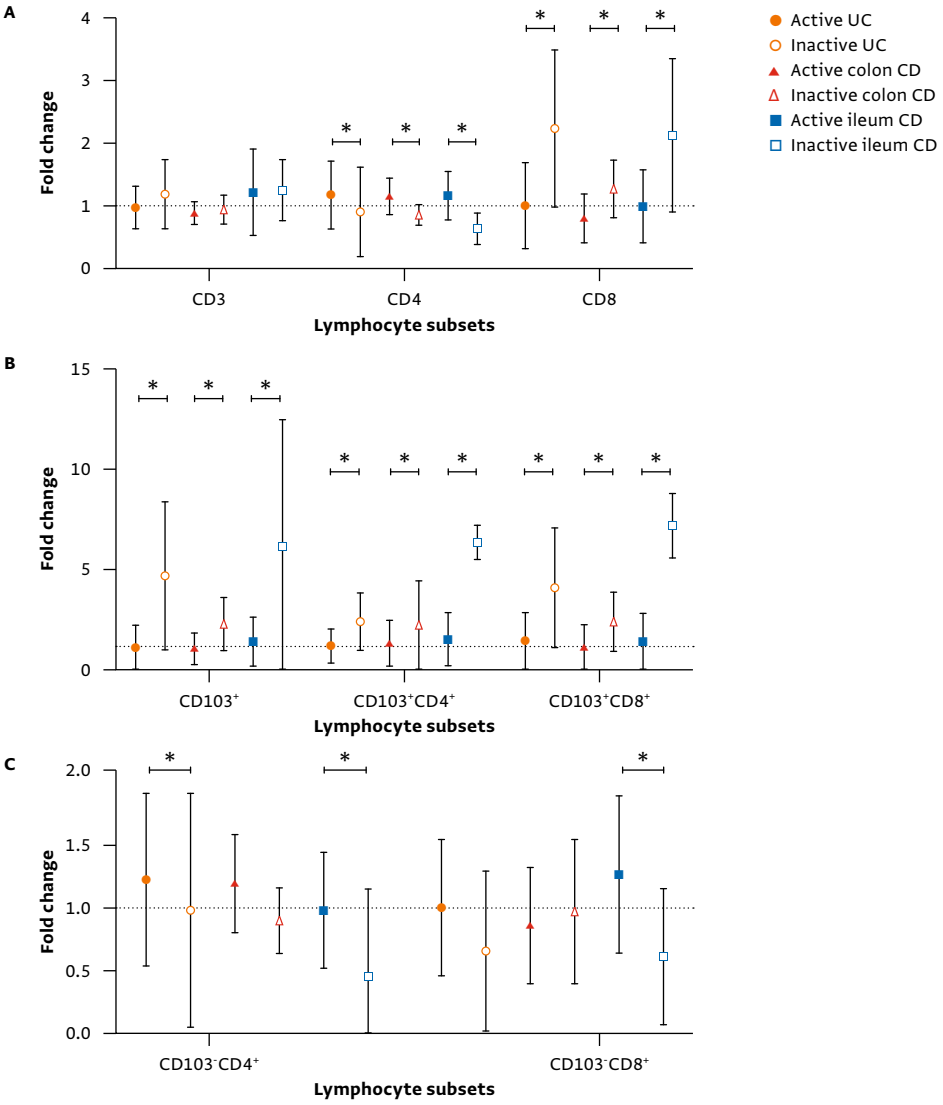
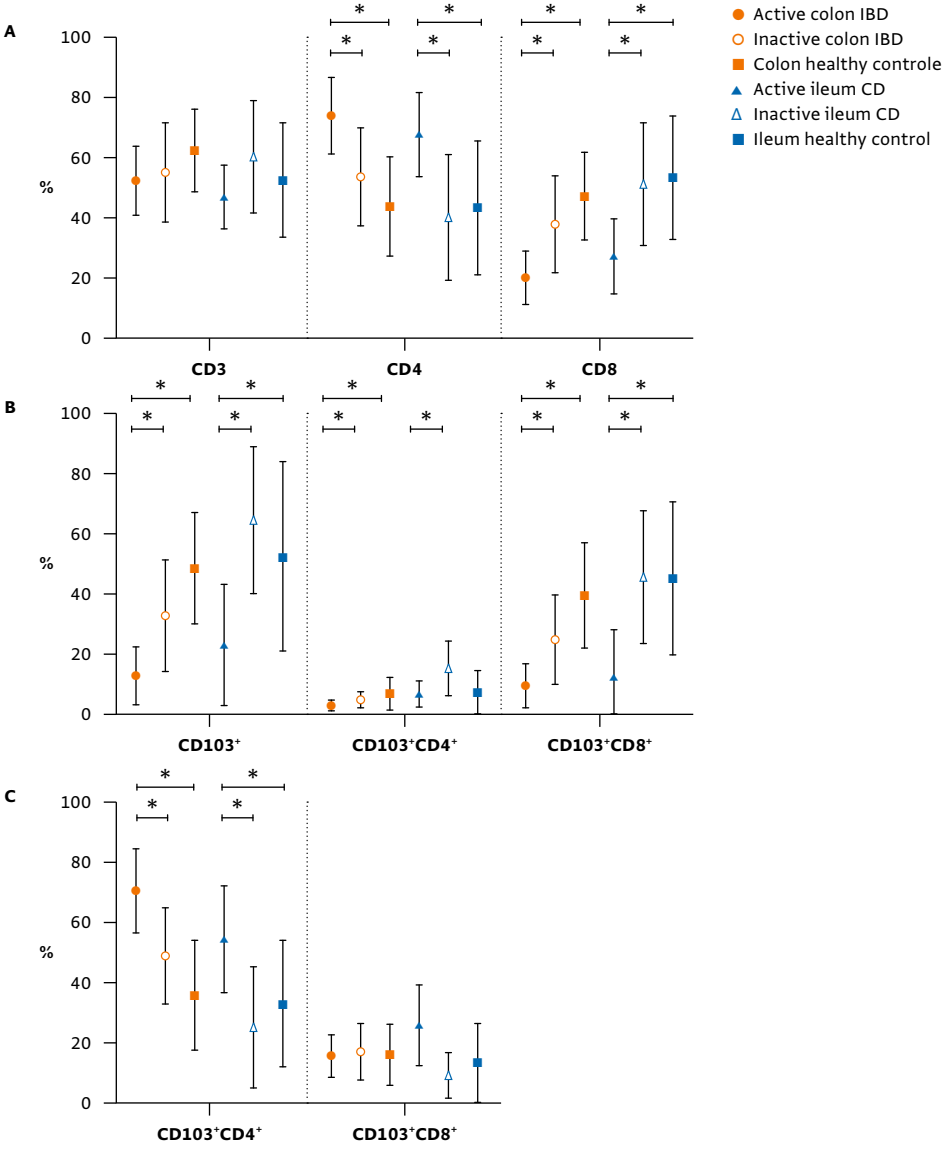


Figure 5 *Percentages of the different intestinal lymphocyte subsets at follow-up endoscopy (A: CD3, CD4, CD8; B: CD103$^+$, CD103$^+$CD4$^+$, CD103$^+$CD8$^+$; C: CD103-CD4$^+$, CD103-CD8$^+$). ● Active colon IBD (n=22) ○ Inactive colon IBD (n=17) ■ Colon healthy control (n=16) ▲ Active ileum CD (n=6) △ Inactive ileum CD (n=17) ■ Ileum healthy control (n=5) * Significant p-value ≤0.05.*



In comparison to the ileum, the percentages of $CD103^+CD4^+$ T-cell subsets in the colonic tissue of CD patients with inactive disease only showed a trend towards levels seen in HC. The % $CD103^+CD8^+$ T cells in the colon increased statistically significant from baseline (p=0.025). The $CD4^+/CD8^+$ ratio in the colon of patients with inactive disease was 2.08 (1.3-2.33), statistically not different from healthy controls (**Figure 3,4,5** and **Supplementary Table 5**). There were also no differences between T-cell subpopulations in colon of CD patients with active disease during follow-up compared to their baseline levels.

Discussion

In the present study, we found substantial differences in the intestinal $CD103^+$ T lymphocyte subsets of patients with active CD and UC compared to HC and patients in endoscopic remission. Percentages of $CD103^-$ T-cell subsets were higher in inflamed ileum and colon of newly diagnosed IBD patients compared to HC and IBD in remission, with the majority consisting of $CD103^-CD4^+$ T cells. Baseline numbers in active disease of both $CD103^+CD4^+$ and $CD103^+CD8^+$ T cells were decreased compared to HC, with a more pronounced decrease in the $CD103^+CD8^+$ subset. The same differences were found in the $CD103^+CD69^+$ T-cell subsets. Percentages of $CD103^+CD4^+$ and $CD103^+CD8^+$ T cells were low in patients with active disease during follow-up endoscopy, comparable to active disease at baseline. In patients with endoscopic remission during follow-up, the proportion of $CD103^+CD4^+$ and $CD103^+CD8^+$ T cells increased to levels comparable to HC.

The intestine of healthy humans comprises almost equal proportions of $CD4^+$ and $CD8^+$ T cells with marginally higher numbers of $CD8^+$ T cells.[21] The majority of intestinal $CD103^+$ T cells belongs to the $CD8^+$ subset (76%).[21] The present results are in line with these findings, as we demonstrated a CD4/CD8 ratio of 0.7-1.2 and a predominance (75-80%) of $CD8^+$ T cells within the $CD103^+$ T-cell subset. CD103 expression is confined to a small subset (<2%) of circulating T lymphocytes, which was confirmed in our cohort.[15,22] After migration to the mucosal tissue, CD103 expression on T cells is induced and maintained by TGF-β, produced by epithelial and dendritic cells.[23] CD103 is expressed at high levels on T cells in the skin, eyes and in the mucosa of the gut and lungs of healthy patients21, but also on dendritic cells, innate lymphoid cells and natural killer cells.[23] Its function is exerted through binding to E-cadherin on intestinal epithelial cells (IEC), mediating cell adhesion and retention of T cells within the mucosa. On the other hand, the IEC themselves are able to interact and activate different T-cell subsets, and were demonstrated to induce a $CD103^+CD8^+$ subset with regulatory function.[24]

In sarcoidosis, reduced frequencies of $CD103^+CD4^+$ and $CD103^+CD8^+$ T cells were found in bronchoalveolar lavage fluid compared to patients with other interstitial lung disease e.g. hypersensitivity pneumonitis, idiopathic pulmonary fibrosis, non-specific interstitial pneumonia and cryptogenic organizing pneumonia.[25] These reduced $CD103^+$

T-cell percentages which are accompanied by a peripheral $CD4^+$ T-cell lymphopenia, suggest a recruitment of $CD4^+$ T cells towards lung tissue.

Other studies on CD103 expression in IBD showed contradictory results. Flow-cytometric analysis by Elewaut et al. showed decreased percentages of $CD103^+$ T cells within intraepithelial lymphocytes (IEL) in ileal and colonic tissue of CD patients compared to HC.[26] No differences in numbers of $CD103^+$ T cells were seen in the lamina propria (LP) of CD patients compared to HC.[26] Analysis with immunohistochemistry in another study showed no differences between IBD and HC in frequencies of epithelial $CD103^+$T cells but demonstrated higher numbers of lamina propria $CD103^+$ T cells in IBD.[27] Using immunohistochemistry and quantitative RT-PCR, Ichikawa et al. showed a higher proportion of αE^+ T cells in the ileum in comparison to the colon of patients with longstanding CD and UC as well as in HC. In CD patients with active colon disease, reduced numbers of the total amount of αE^+ T cells were seen compared to HC and UC patients. Overall, αE levels were not affected by inflammation and did not differ from HC.[28]

Regarding different $CD103^+$ T-cell subsets in IBD, higher percentages of intestinal $CD103^+CD4^+$ subsets were recently described by performing immunohistochemistry in a small number of UC and CD patients versus HC. No difference was found for the percentages of $CD103^+CD8^+$ T cells, in neither epithelium nor lamina propria of IBD patients compared to HC.[11] However, these patients were under treatment with anti-$\alpha4\beta7$ antibodies (Vedolizumab), which could have influenced the frequencies of $\beta7^+$ T-cell subsets in gut mucosa and peripheral blood. Therefore these findings are not comparable with our results in untreated patients. Further experiments in DSS mice models by the same authors demonstrated an inhibitory effect of treatment with anti-β7 antibody (Etrolizumab) on the accumulation of $CD103^+CD8^+$, but not on the $CD103^+CD4^+$ T cells in the mucosa.[11] Nevertheless, the percentage of $CD103^+CD4^+$ of all intestinal $CD103^+$ T cells has been reported to be very low; the majority of the $CD103^+$ T cells is $CD8^+$.[29] In above mentioned studies12,26,27,28, inflamed and uninflamed biopsies of long-standing IBD patients under treatment were statistically analysed together, which could have influenced the numbers of αE^+ T cells. The discrepancies between studies can also be explained by the method used (immunohistochemistry versus FACS) and the number of included patients.

The protein CD69 is a marker for early lymphocyte activation and tissue retention.[14] We observed however that T cells with a TRM phenotype ($CD103^+$ and $CD69^+$) were present in lower percentages in untreated newly diagnosed IBD patients compared to patients with inactive disease and HC. It is mainly the $CD103^-(CD4^+)$ T cell population that is clearly increased in active IBD. Expression of CD69 which also may suggest activation was not different on this $CD103^-CD4^+$ T cell population in active IBD compared to HC.

In a TNF-driven mice model of chronic ileitis, $CD103^+CD8^+$ T cells exerted regulatory functions by producing TGF-β, inhibiting proliferation of $CD4^+$ T cells and attenuating transferred ileitis in vivo.[30] Another study displayed an essential role in mucosal im-

mune regulation for $CD103^+$ T cells explained by regulatory T cell-mediated suppression of colitis, which was absent in $CD103^-$deficient mice.[31] Recently, a pro-inflammatory role has been suggested for the $CD103^+CD4^+$ T cells in IBD explained by expression of higher levels of IFNγ and TNFα, while this could not be demonstrated for $CD103^+CD8^+$T cells.[12] The functional features of $CD103^+CD8^+$ are in need of further investigation in the future, as this subset has been demonstrated to possess substantial regulatory capacities in mice models.

In the present study, frequencies of $CD103^+CD4^+$ and $CD103^+CD8^+$ T-cell subsets are described for the first time in a large cohort of newly diagnosed patients, in inflamed and non-inflamed ileum and colon samples analysed separately, at baseline and during follow-up. Our results are in line with previous studies showing that $CD103^+$ T cells in colon of HC and of IBD patients in remission mainly consists of $CD8^+$ cells.[21] The largest intestinal T-cell subset involved in active IBD is the $CD103^-CD4^+$ subset. These higher numbers of $CD103^-$ T cells next to their low proliferation level (Ki-67 fluorescence intensity) confirm rather a redistribution from peripheral origin of this subset instead of a local proliferation. Furthermore, in our patients, frequencies of both $CD103^+CD4^+$ and $CD103^+CD8^+$ subsets did not increase in the inflamed gut mucosa during follow-up endoscopy. Therefore these findings do not underline an upregulation of CD103 on intestinal T cells in the inflamed gut mucosa, in time.

The proportion of $CD103^+$ T cells, $CD4^+$ as well as $CD8^+$ subsets increased in our patients reaching endoscopic remission. We can also confirm that CD patients in remission and HC express higher percentages of $CD103^+$ T cells in the ileum mucosa compared to the colon. This is in line with previous studies describing a linear decrease in the number of T cells expressing CD103 from the ileum and ascending colon to rectum.[28,32]

A limitation of the present study might be the use of intestinal biopsies that do not reach the deepest layers of the intestinal wall such as the muscularis layer. This might lead to an underestimation of the T-cell infiltration of the deepest layers in CD patients with transmural inflammation. The mechanical method used to pre-process the biopsy specimens before flowcytometry might have had an influence on the absolute cell numbers. However in a recent comparison of different methods (mechanical, enzymatic and organ culture protocols), they all proved to have their limitations33. In line with this method, we were not able to distinguish the lymphocyte infiltrate in lamina propria from epithelium.

In conclusion, $CD103^+CD4^+$ and $CD103^+CD8^+$ subsets represent only a minority of the T-cell infiltrate in the inflamed gut and maintenance of these low numbers was seen in active disease during follow-up. The majority of the mucosal infiltrating T cells in active IBD consists of the $CD103^-CD4^+$ subset both at diagnosis and follow-up. Therefore, we found no evidence for an upregulation of $CD103^+$ on intestinal $CD4^+$ and $CD8^+$ T cells during chronic inflammation in time.

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Supplementary material

Figure S1 *Baseline endoscopic inflammation in UC (A) and CD (B) patients is not correlated with percentages of different CD103⁺ T-cell subsets. UC, ulcerative colitis; CD, Crohn's disease; SES-CD, Simple Endoscopic Score for Crohn's Disease.*

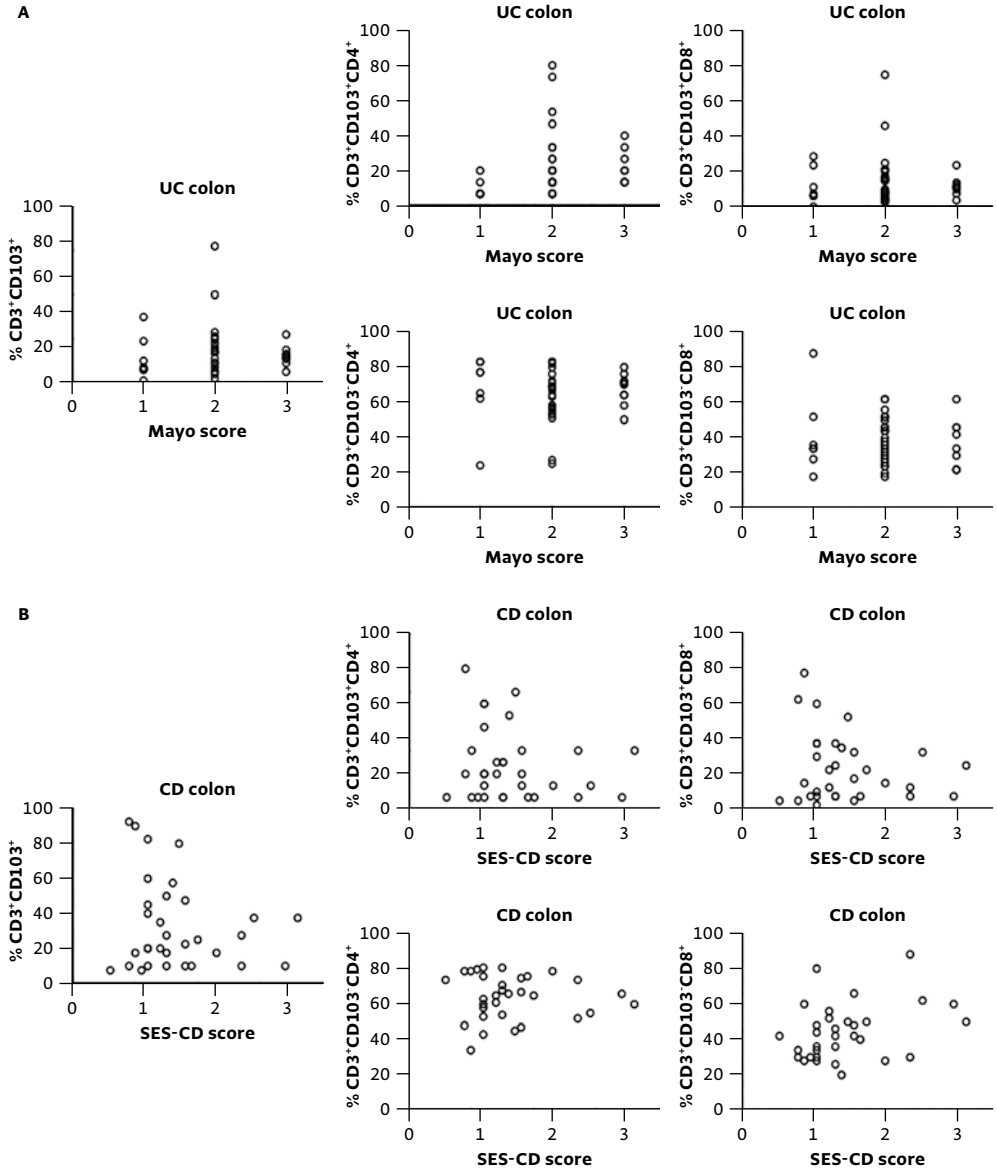

Table S1 *Fluorochromes used for Flow Cytrometry (all obtained from Becton Dickinson Biosciences USA).*

T-cell marker	**CD3**	**CD4**	**CD8**	**CD103**	**CD45RA**	**CD27**
Fluorochrome	APC	PE/Cy7	APC H7/Cy7	FITC	PE	PerCP/Cy5.5
T-cell marker	CD69	Ki-67	FoxP3	CD25		
Fluorochrome	PE or PerCP/Cy5.5	PE	PE	APC		

Table S2 *Baseline T-cell subsets in Crohn's disease, ulcerative colitis and healthy controls, median percentages (IQR). CD, Crohn's disease, UC, Ulcerative Colitis, IQR, Interquartile range. * Significant p-value ≤0.05.*

	CD	**UC**	**HC**	**p-value**		
Location of biopsies inflamed mucosa	**Colon (n=49)**	**Ileum (n=43)**	**Colon (n=49)**	**Colon (n=16)**	**Ileum (n=5)**	
CD3$^+$% ∫	58 (49-68)	50 (39-62)	54 (47-67)	62 (57-70)	62 (38-73)	[1]0.266 [2]0.128 [3]0.322 40.493
CD3$^+$CD4$^+$% †	70 (63-74)	68 (58-77)	72 (62-80)	39 (31-56)	55 (20-61)	[1]0.001* [2]0.001* [3]0.012* [4]0.281
CD3$^+$CD8$^+$% †	28 (22-32)	26 (20-32)	23 (18-34)	50 (36-56)	45 (36-75)	[1]0.001* [2]0.001* [3]0.003* [4]0.269
CD3$^+$CD103$^+$% †	11 (7-21)	13 (7-31)	11 (6-20)	52 (34-61)	40 (26-86)	[1]0.001* [2]0.001* [3]0.012* [4]0.746
CD3$^+$CD103$^+$CD8$^+$% †	9 (4-15)	9 (5-24)	9 (5-14)	42 (23-57)	33 (24-73)	[1]0.001* [2]0.001* [3]0.005* [4]0.859
CD3$^+$CD103$^-$CD8$^+$% †	22 (15-27)	17 (13-26)	19 (15-23)	13 (10-20)	9 (5-24)	[1]0.001* [2]0.017* [3]0.092 40.106
CD3$^+$CD103$^+$CD4$^+$% †	3 (1-5)	4 (2-9)	3 (1-4)	5 (5-7)	3 (2-14)	[1]0.007* [2]0.002* [3]0.844 [4]0.664

$CD3^+CD103^-CD4^+$% †	65 (52-74)	62 (44-74)	69 (58-77)	30 (21-50)	35 (9-56)	[1]0.001* [2]0.001* [3]0.020* [4]0.091
$CD3^+CD103^+CD8^+/CD8^+$ ratio	0.33 (0.18-0.54)	0.39 (0.23-0.69)	0.39 (0.25-0.47)	0.83 (0.59-1.04)	0.80 (0.64-0.98)	[1]0.001* [2]0.001* [3]0.013* [4]0.597
$CD3^+CD103^-CD8^+/CD8^+$ ratio	0.86 (0.66-1.0)	0.74 (0.53-1.1)	0.79 (0.64-0.98)	0.33 (0.22-0.46)	0.22 (0.06-0.6)	[1]0.001* [2]0.001* [3]0.009* [4]0.541
$CD3^+CD103^+CD4^+/CD4^+$ ratio	0.04 (0.01-0.08)	0.06 (0.02-0.16)	0.03 (0.02-0.07)	0.11 (0.08-0.21)	0.13 (0.03-0.51)	[1]0.001* [2]0.001* [3]0.392 [4]0.497
$CD3^+CD103^-CD4^+/CD4^+$ ratio	0.92 (0.85-0.97)	0.9 (0.8-0.97)	0.95 (0.90-0.99)	0.82 (0.68-0.91)	0.81 (0.42-0.92)	[1]0.007* [2]0.001* [3]0.100 [4]0.073
$CD4^+/CD8^+$ ratio	2.4 (1.9-3.5)	2.8 (1.7-3.8)	3.0 (2.0-4.2)	0.7 (0.6-1.5)	1.2 (0.27-1.7)	[1]0.001* [2]0.001* [3]0.005* [4]0.215

1 P-value comparison active CD colon with HC colon.
2 P-value comparison active UC colon with HC colon.
3 P-value comparison active CD ileum with HC ileum.
4 P-value comparison active CD colon with active UC colon.
∫ Percentages within all lymphocytes
† Percentages within $CD3^+$ T cells

Table S3 *Baseline % of the different $CD103^+$ T-cell subsets within all lymphocytes in CD and UC patients in PBMC (peripheral blood mononuclear cells), median percentage (IQR). CD, Crohn's disease, UC, Ulcerative Colitis, IQR, Interquartile range.*

	UC (n=49)	**CD (n=75)**	**HC (n=16)**
$CD3^+CD103^+$%	1.0 (0.7-1.0)	1.0 (0.5-1.0)	1.0 (0.7-1.0)
$CD3^+CD103^+CD8^+$%	0.45 (0.3-0.7)	0.3 (0.1-0.7)	0.3 (0.1-0.6)
$CD3^+CD103^+CD4^+$%	0.3 (0.2-0.4)	0.3 (0.2-0.5)	0.3 (0.2-0.4)

Table S4 *Follow-up % of T-cell subsets in UC patients in remission and in active disease, medians (IQR). UC, Ulcerative Colitis, IQR, Interquartile range. * Significant p-value ≤0.05.*

	UC-remission	UC-active	p-value*
Location of biopsies inflamed mucosa	**Colon (n=7)**	**Colon (n=13)**	
$CD3^+$% ∫	55 (46-85)	52 (40-59)	[1]0.535 [2]0.398 [3]0.345 [4]0.351
$CD3^+CD4^+$% †	51 (39-66)	77 (74-85)	[1]0.376 [2]0.236 [3]0.423 [4]0.001*
$CD3^+CD8^+$% †	41 (25-54)	19 (11-24)	[1]0.535 [2]0.128 [3]0.388 [4]0.001*
$CD3^+CD103^+$% †	46 (22-58)	8 (3-14)	[1]0.413 [2]0.024 [3]0.484 [4]0.001*
$CD3^+CD103^+CD8^+$% †	38 (18-45)	6 (2-13)	[1]0.376 [2]0.034* [3]0.463 [4]0.002*
$CD3^+CD103^-CD8^+$% †	10 (9-19)	14 (10-19)	[1]0.871 [2]0.310 [3]0.576 [4]0.700
$CD3^+CD103^+CD4^+$% †	5 (3-8)	3 (1-4)	[1]0.579 [2]0.016* [3]0.564 [4]0.011
$CD3^+CD103^-CD4^+$% †	45 (31-65)	76 (69-86)	[1]0.341 [2]0.237 [3]0.345 [4]0.001*
$CD3^+CD103^+CD8^+/CD8^+$ ratio	0.77 (0.72-0.83)	0.43 (0.13-0.64)	[1]0.452 [2]0.012* [3]0.701 [4]0.019*

$CD3^+CD103^-CD8^+/CD8^+$ ratio	0.33 (0.22-0.46)	0.92 (0.61-1.14)	[1]0.974 [2]0.237 [3]0.807 [4]0.046*
$CD3^+CD103^+CD4^+/CD4^+$ ratio	0.13 (0.06-0.25)	0.04 (0.01-0.05)	[1]0.871 [2]0.063 [3]0.972 [4]0.001*
$CD3^+CD103^-CD4^+/CD4^+$ ratio	0.82 (0.68-0.91)	0.99 (0.96-1.0)	[1]0.135 [2]1.000 [3]0.133 [4]0.019*
$CD4^+/CD8^+$ ratio	0.7 (0.57-1.53)	4.05 (3.17-8.12)	[1]0.376 [2]0.063 [3]0.272 [4]0.001*

1 P-value comparison UC in endoscopic remission with HC.
2 P-value comparison UC in endoscopic remission at follow-up with their own baseline values during active UC.
3 P-value comparison endoscopic active UC at follow-up with their own baseline values during active UC.
4 P-value comparison endoscopic active UC at follow-up with endoscopic inactive UC at follow-up.
∫ Percentages within all lymphocytes.
† Percentages within $CD3^+$ T cells.

Table S5 *Follow-up % of T-cell subsets in Crohn's disease in remission and in active disease, medians (IQR). CD, Crohn's disease, IQR, Interquartile range. * Significant p-value ≤0.05.*

	CD-remission	**P-value***	**CD-active**	**p-value****		
Location of biopsies inflamed mucosa	**Colon (n=10)**	**Ileum (n=17)**		**Colon (n=9)**	**Ileum (n=6)**	
$CD3^+$%∫	51 (42-59)	60 (46-69)	[1]0.053 [2]0.940 [3]0.213 [4]0.201	50 (48-68)	45 (38-58)	[5]0.169 [6]0.329 [7]0.141 [8]0.753
$CD3^+CD4^+$%†	62 (48-66)	40 (30-48)	[1]0.014* [2]0.949 [3]0.016* [4]0.001*	62 (56-82)	71 (56-80)	[5]0.002* [6]0.052 [7]0.123 [8]0.463
$CD3^+CD8^+$%†	30 (27-37)	53 (42-65)	[1]0.017* [2]0.940 [3]0.092 [4]0.003*	26 (15-31)	25 (17-38)	[5]0.001* [6]0.052 [7]0.093 [8]0.588
$CD3^+CD103^+$%†	26 (18-35)	72 (57-79)	[1]0.014* [2]0.649 [3]0.025* [4]0.001*	20 (8-26)	17 (8-44)	[5]0.001* [6]0.126 [7]0.674 [8]0.463

$CD3^+CD103^+CD8^+$%†	20 (14-25)	48 (37-59)	[1]0.005* [2]1.000 [3]0.025* [4]0.002*	15 (6-22)	7 (4-21)	[5]0.001* [6]0.030* [7]0.528 [8]0.684
$CD3^+CD103^-CD8^+$%†	17 (13-26)	7 (3-15)	[1]0.220 [2]0.401 [3]0.440 [4]0.006*	20 (10-22)	25 (15-37)	[5]0.388 [6]0.177 [7]0.159 [8]0.528
$CD3^+CD103^+CD4^+$%†	5 (3-6)	13 (9-21)	[1]0.310 [2]0.039* [3]0.838 [4]0.003*	4 (3-6)	7 (3-11)	[5]0.136 [6]0.662 [7]0.916 [8]0.753
$CD3^+CD1035CD4^+$%†	54 (47-60)	18 (15-28)	[1]0.014* [2]0.704 [3]0.139 [4]0.002*	60 (50-74)	50 (39-72)	[5]0.002* [6]0.177 [7]0.263 [8]0.345
$CD3^+CD103^+CD8^+/CD8^+$ ratio	0.59 (0.5-0.73)	0.96 (0.64-1.0)	[1]0.027* [2]0.395 [3]0.074 [4]0.019*	0.59 (0.21-0.80)	0.35 (0.15-0.59)	[5]0.049* [6]0.030* [7]0.889 [8]0.753
$CD3^+CD103^-CD8^+/CD8^+$ ratio	0.56 (0.32-0.83)	0.13 (0.05-0.28)	[1]0.041* [2]0.704 [3]0.114 [4]0.031*	0.8 (0.58-0.93)	1.11 (0.68-1.46)	[5]0.001* [6]0.030* [7]1.000 [8]0.116
$CD3^+CD103^+CD4^+/CD4^+$ ratio	0.08 (0.04-0.138)	0.43 (0.31-0.66)	[1]0.135 [2]0.140 [3]0.386 [4]0.001*	0.06 (0.04-0.09)	0.1 (0.04-0.2)	[5]0.017* [6]0.792 [7]0.779 [8]0.917
$CD3^+CD103^-CD4^+/CD4^+$ ratio	0.94 (0.84-0.98)	0.58 (0.45-0.64)	[1]0.047* [2]0.319 [3]0.333 [4]0.006*	0.96 (0.83- 0.99)	0.76 (0.66-0.96)	[5]0.032* [6]0.537 [7]0.263 [8]0.046
$CD4^+/CD8^+$ ratio	2.08 (1.3-2.33)	0.73 (0.46-1.15)	[1]0.020* [2]0.762 [3]0.093 [4]0.023*	2.48 (1.81-5.71)	3.0 (1.54-4.8)	[5]0.001* [6]0.052 [7]0.093 [8]0.463

1 P-value comparison CD colon in endoscopic remission with HC colon.
2 P-value comparison CD ileum in endoscopic remission with HC ileum.
3 P-value comparison CD colon in endoscopic remission with their own baseline values during active disease.
4 P-value comparison CD ileum in endoscopic remission with their own baseline value during active disease.
5 P-value comparison CD colon in endoscopic active disease with HC colon.
6 P-value comparison CD ileum in endoscopic active disease with HC ileum.
7 P-value comparison CD colon in endoscopic active disease with their own baseline values during active disease.
8 P-value comparison CD ileum in endoscopic active disease with their own baseline value during active disease.
∫ Percentages within all lymphocytes.
† Percentages within $CD3^+$ T cells.

CHAPTER 5

Homeostatic function and inflammatory activation of ileal CD8$^+$ tissue-resident T cells is dependent on mucosal location

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Lisanne Lutter

Britt Roosenboom

Eelco C. Brand

José J. ter Linde

Bas Oldenburg

Ellen G. van Lochem

Carmen S. Horjus Talabur Horje

Femke van Wijk

Abstract

BACKGROUND & AIMS Tissue-resident memory T (Trm) cells, both of the CD4 and CD8 lineage, have been implicated in disease flares in inflammatory bowel disease. However, data are conflicting regarding the profile of human $CD8^+$ Trm cells, with studies suggesting both proinflammatory and regulatory functions. It is crucial to understand the functional profile of these cells in the context of (new) therapeutic strategies targeting (trafficking of) gut Trm cells.

METHODS Here, we performed imaging mass cytometry, flow cytometry and RNA-sequencing to compare lamina propria and intraepithelial $CD103^+$/-$CD69^+CD8^+$ T cells in healthy control subjects and patients with active ileal Crohn's disease.

RESULTS Our data revealed that lamina propria $CD103^+CD69^+CD8^+$ T cells have a classical Trm profile with active pathways for regulating cell survival/death and cytokine signaling, whereas intraepithelial $CD103^+CD69^+CD8^+$ T cells display a tightly regulated innate-like cytotoxic profile. Furthermore, within lamina propria $CD8^+CD103^-$ Trm cells, an $Itgb2^+GzmK^+KLRG1^+$ population distinct from $CD103^+$ $CD8^+$ Trm is found. During chronic inflammation, especially intraepithelial $CD103^+CD69^+CD8^+$ T cells displayed an innate proinflammatory profile with concurrent loss of homeostatic functions.

CONCLUSIONS Altogether, these compartmental and inflammation-induced differences indicate that therapeutic strategies could have a different impact on the same immune cells depending on the local compartment and presence of an inflammatory milieu, and should be taken into account when investigating short- and long-term effects of new gut T cell-targeting drugs.

Introduction

Inflammatory bowel disease (IBD), comprising Crohn's disease (CD) and ulcerative colitis, is a chronic relapsing-remitting inflammatory disease. To date, there is no cure for IBD; therefore, long-term administration of maintenance therapy is often necessary. Recently, a novel class of drugs has been added to the therapeutic armamentarium for IBD, namely compounds that modulate lymphocyte trafficking such as vedolizumab (anti-integrin α4β7) and natalizumab (anti-integrin α4). Another anti-integrin, etrolizumab (anti-integrin β7), is currently in phase III trials.[1,2] Expression of integrins enables homing of immune cells to tissues, with integrin α4β7 being the primary gut homing receptor.[3] Upon localization to the gut the integrin β7 monomer can dimerize with integrin αE (CD103). Upregulation of CD103 enables T cells to bind to E-cadherin, expressed by epithelial cells, thereby facilitating their intraepithelial retention.[4-6] $CD4^+$ T cells are more abundant in the lamina propria, while T cells in the epithelium are primarily of the $CD8^+$ lineage.[4] T cells homed to the lamina propria and epithelium can become tissue-resident memory T (Trm) cells upon expression of the Trm cell markers CD69 and CD103.[7] Local cues, distinct for the lamina propria and epithelium, might induce further environment-adapted specialization of these Trm cells ($CD69^+CD103^{+/-}$).[8-10]

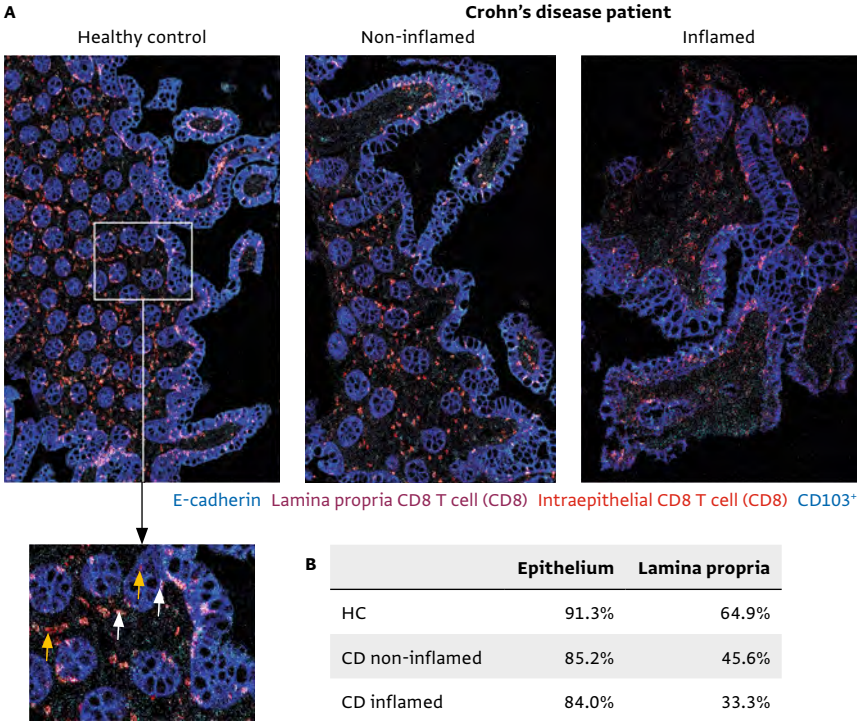
Recently, it has been suggested that lamina propria $CD4^+$ and $CD8^+$ $CD69^+CD103^+$ Trm might be implicated in disease flares in IBD,11 which implies that targeting these cells in IBD could be beneficial. A proinflammatory profile of colonic $CD4^+CD103^+$ T cells in IBD flares has been observed,12,13 but the functional profile of intestinal $CD8^+CD103^+$ T cells is still not completely elucidated.[14-16] Interestingly, in mice adoptive transfer of $CD8^+CD103^+$ T cells reduced the severity of ileitis.[17] Furthermore, we have previously shown that mucosal $CD8^+CD103^+$ T cell percentages in humans decrease by approximately 40% during CD flares compared to healthy control subjects, and normalize upon achieving remission.[18,19] These findings raise the question whether these cells could have a proinflammatory or regulatory function. To determine compartmental differences and the functional profile of intestinal $CD8^+$ Trm cells, we performed flow cytometry, imaging mass cytometry (IMC) and RNA-sequencing on lamina propria and intraepithelial $CD103^+$ (and $CD103^-$) $CD69^+CD8^+$ T cells in healthy control subjects and patients with active ileal CD.

Results

We determined the localization of ileal $CD8^+CD103^+$ T cells in healthy control subjects and patients with *de novo* CD with imaging mass cytometry. We observed a decrease in percentage of $CD103^+$ cells of total $CD8^+$ T cells in both the epithelium and lamina propria of CD patients compared to healthy control ileum (**Figure 1A**). This decrease was most pronounced in the lamina propria with, on average, a 50% reduction in $CD103^+$

CD8$^+$ T cells compared to a 10% decrease in the epithelium (**Figure 1B**). Upon presence of inflammation in CD patients, there was an additional 30% decrease in CD103$^+$ CD8 T cells in the lamina propria, whereas in the epithelium CD8$^+$ T cells remained predominantly CD103$^+$ (**Figure 1B**). Furthermore, there was an absolute decrease in CD8$^+$ and consequently CD8$^+$CD103$^+$ T cells per µm2 in both the epithelium and lamina propria of CD patients compared with healthy control subjects (average of 1 CD8$^+$ T cell per 1051 µm2 in human control subjects, per 2249 µm2 in noninflamed CD patients, and per 2589 µm2 in inflamed CD patients for the epithelium, and per 839, 1848, and 1957 µm2, respectively, for the lamina propria).

Figure 1 *Visualization of CD103$^-$ and CD103$^+$ CD8$^+$ T cells in the human ileum. (A) Representative composite images of imaging mass cytometry performed on human ileum sections showing an overlay of E-cadherin (blue), CD8 (red in lamina propria, pink in epithelium), and CD103 (cyan, colors white in CD8 T cells) for a healthy control (HC) subject (left), noninflamed ileum CD patient (middle), and inflamed ileum of a CD patient (right). The magnified section of the HC subject shows an example of CD103$^+$ (white arrows) and CD103$^-$ (orange arrows) CD8$^+$ T cells within the lamina propria and epithelium. HC subjects: n = 2; CD patients: n = 3 (paired). (B) Quantification of CD103$^+$ within CD8$^+$ T cells in both the epithelium and lamina propria of the HC, CD noninflamed, and CD inflamed ileum. Every value is an average of 2 (HC subjects and noninflamed CD patients) or 3 (inflamed CD patients) samples measured using imaging mass cytometry. For each of the samples, 2 independent counts were performed.*



B

	Epithelium	Lamina propria
HC	91.3%	64.9%
CD non-inflamed	85.2%	45.6%
CD inflamed	84.0%	33.3%

Flow cytometry analysis of ileal CD8$^+$ Trm cells in untreated, *de novo* CD (n = 21) (**Figure 2A**) showed a negative trend between the proportion of CD8$^+$CD69$^+$CD103$^+$ T cells and the simple endoscopic score for CD of the ileum (**Figure 2B**). No correlation with other clinical parameters including the Harvey-Bradshaw index, fecal calprotectin or C-reactive protein was found. In addition, we observed a higher proportion of dividing CD8$^+$CD69$^+$CD103$^+$ compared to CD103$^-$ T cells in these untreated, *de novo* CD patients (average of 16.4% and 4.5% Ki-67$^+$ cells, respectively) (**Figure 2C**), indicating that CD8$^+$CD69$^+$CD103$^+$ T cells are activated during inflammation.

To investigate the transcriptional profile of gut Trm cells, we performed RNA-sequencing on flow cytometry-based sorted lamina propria and intraepithelial CD103$^+$ (and CD103$^-$) CD69$^+$CD8$^+$ T cells in healthy control subjects and patients with active ileal CD. Unsupervised principal component analysis revealed that samples primarily cluster based on the compartment of residence. This relative compartmentalization was less evident during inflammation and for CD103$^-$ T cells (**Figure 2D**).

Lamina propria CD8$^+$CD69$^+$CD103$^-$ and CD8$^+$CD69$^+$CD103$^+$ T cells have distinct profiles

We first compared the transcriptional profile of CD103$^+$ and CD103$^-$ CD8$^+$ Trm cells (CD69$^+$) in the lamina propria. Differential gene expression revealed 22 upregulated and 54 downregulated genes between CD103$^+$ and CD103$^-$ CD8$^+$ Trm cells (false discovery rate [FDR] <0.1) (**Supplementary Table 1**). These were shared by inflamed (CD) and non-inflamed (healthy control) ileum. *KLF2*, *ENC1*, *GZMK*, *KLRG1*, and *S1PR5* genes known to be downregulated in Trm cells7 were also downregulated in CD103$^+$CD8$^+$ T cells compared with CD103$^-$CD8$^+$ T cells, whereas *EOMES*, a T effector memory-associated transcription factor,20 was upregulated in CD103$^-$CD8$^+$ T cells (**Figure 3A**). In line with a more differentiated Trm cell phenotype, CD103$^+$ CD8$^+$ Trm cells also expressed higher levels of *CD160*, *CD96*, and *KLRC2* (encoding NKG2C) (**Figure 3A**). On the protein level, a lower expression of integrin β2 (Itgb2), GzmK, KLRG1 and EOMES on CD8$^+$CD103$^+$ T cells compared to CD8$^+$CD103$^-$ T cells was confirmed (**Figure 3B**). These data indicate that CD103$^+$ CD8$^+$CD69$^+$ lamina propria T cells express a less cytotoxic but more pronounced classical Trm cell profile compared with their CD103$^-$ counterpart.

Within the CD8$^+$CD69$^+$CD103$^-$ Trm cell compartment, a relatively high heterogeneity was found based on the protein expression data, with <50% Itgb2high, GzmK and KLRG1 expressing cells (**Figure 3B**). Itgb2high expression was almost mutually exclusive with CD103 expression (**Figure 4A**), and expression of GzmK and KLRG1 was predominantly confined to the Itgb2high subset (**Figure 4B** and **C**). Similarly to CD103$^+$ CD8$^+$CD69$^+$ Trm cells, CD8$^+$ Trm cells lacking CD103 and Itgb2high were mostly GzmK and KLRG1 negative (on average 91.8% and 94.2%, respectively; **Figure 4C**). Additionally, PD-1 expression, often associated with clonal expansion of CD8$^+$ T cells,21,22 was higher in the Itgb2high

Figure 2 *Characterizing human intestinal CD103⁻ and CD103⁺ CD69⁺CD8⁺ T cells. (A) Distribution of CD69⁺/- and CD103⁺/⁻ CD3⁺CD8⁺ T-cells within the ileum of patients with active CD (n = 21) at time of endoscopy, characterized by flow cytometry. (B) Scatterplot and fitted linear regression of the simple endoscopic score for CD (SES-CD) for the ileum and the percentage of total mucosal CD8⁺CD69⁺CD103⁺ T cells derived from inflamed ileum of patients with active CD (n = 21), characterized by flow cytometry. Pearson's r and the corresponding P value are depicted in the graph. (C) Representative gating strategy (upper) and quantification (lower) of Ki-67 in both CD103⁻ and CD103⁺ CD69⁺CD8⁺ T cells in patients with active ileal CD (n = 4). The bar represents the median. Comparison was performed with a paired 2-tailed t test. (D) Unsupervised principal component analysis of all CD69⁺CD8⁺ T cell subsets analyzed by RNA-sequencing; CD103- from the lamina propria (orange/circle) and CD103⁺ from the lamina propria (blue/square) and epithelium (red/triangle) from both healthy control (HC) subjects (closed symbols) and CD patients (open symbols). PC, principal component.*

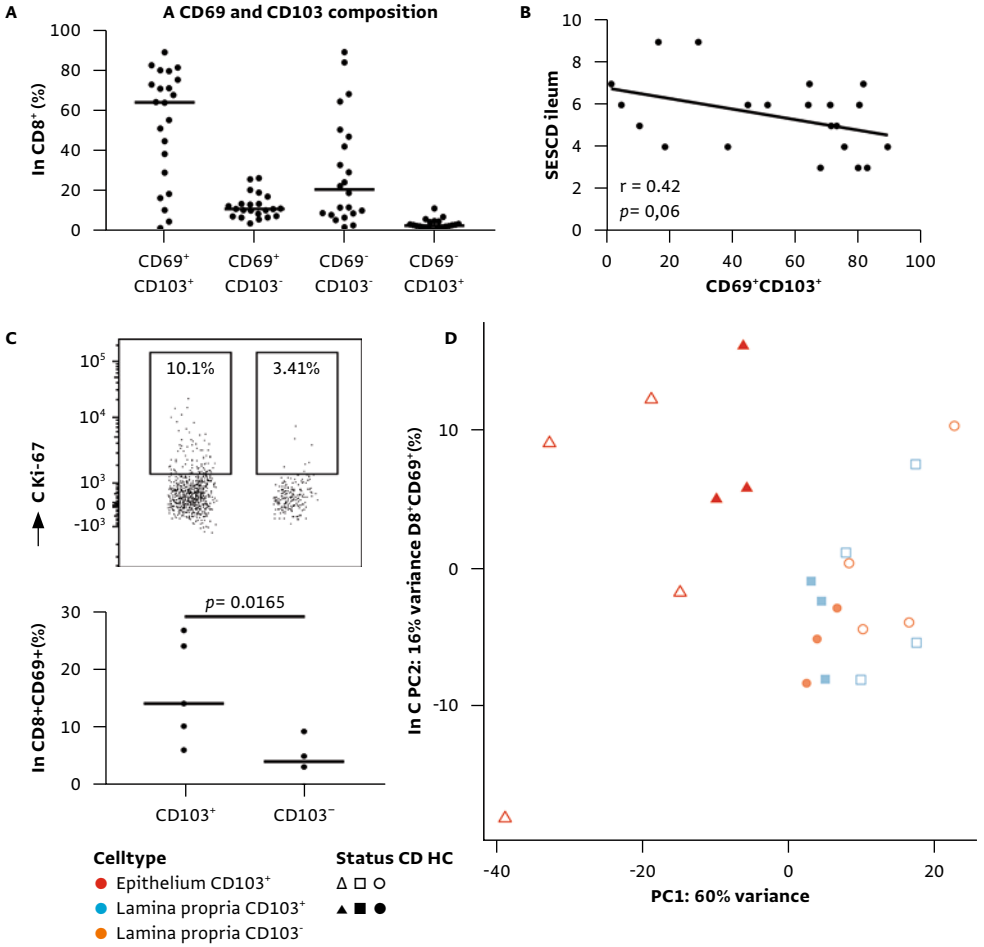
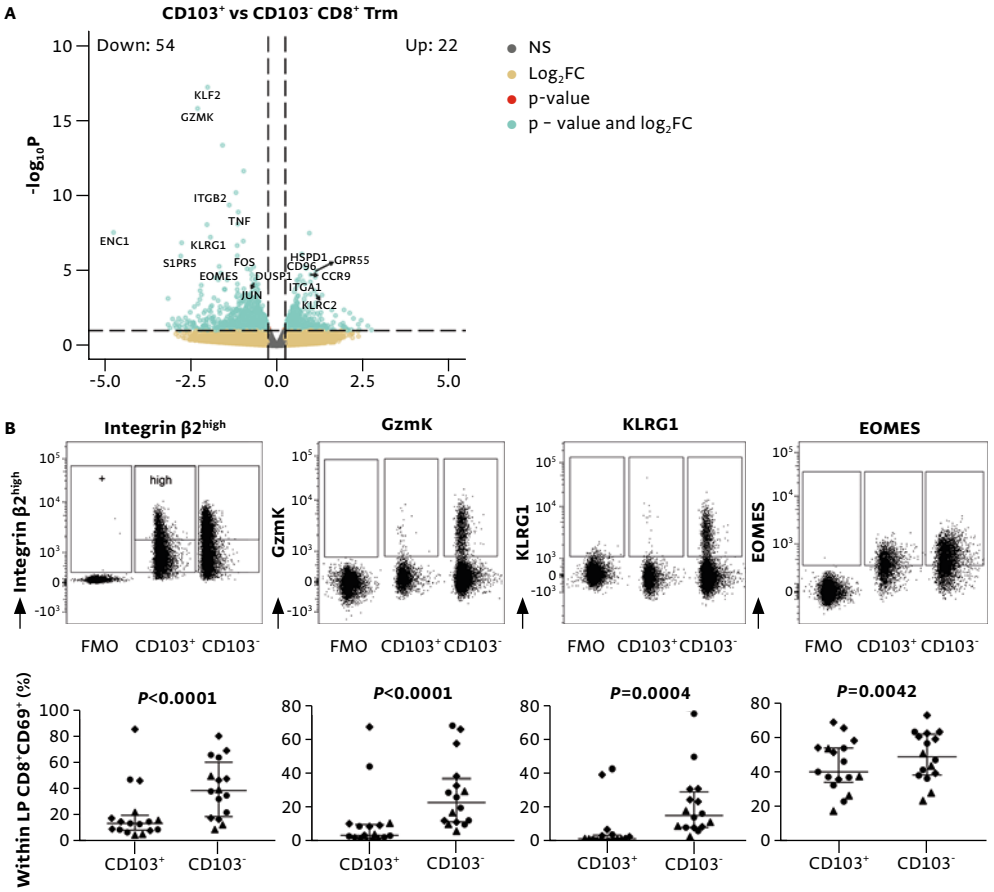


Figure 3 *Subset defining genes of lamina propria CD8⁺CD69⁺CD103⁻ and CD8⁺CD69⁺CD103⁺ T cells. (A) Volcano plot of the expressed genes, with a nominal P value <0.99, comparing lamina propria CD8⁺CD69⁺CD103⁺ to CD8⁺CD69⁺CD103⁻ T cells; selected genes are highlighted. On the x-axis the log2 fold change (log2FC) is shown, and on the y-axis the -log10 P value (-log10P). Grey indicates not significantly differentially expressed genes, yellow indicates genes with a log2FC >0.25 and -log10P > 10 × 10-2.5, green indicates genes with a log2FC >0.25 and -log10P < 10 × 10-2.5. (B) Representative flowcytometric dotplots, including Fluorescent Minus One (FMO) control, of Itgb2, GzmK, KLRG1 and EOMES (upper row) and quantification of the respective marker (lower row) comparing lamina propria CD8⁺CD69⁺CD103⁺ and CD103⁻ T cells in healthy control subjects (n = 6-7; circles), CD from inflamed (diamonds) and noninflamed (triangles) ileum (paired, n = 4-6). Bars represent median and interquartile range. Comparison was performed with a paired 1-tailed t test. NS, not significant.*



compared to CD103⁺ CD8⁺ Trm cells (average of 32.7 vs 14.7%) (**Figure 4D**). In summary, within CD103⁻ CD8⁺ Trm cells an Itgb2high Trm cell population characterized by GzmK, KLRG1 and PD-1 is found.

The dichotomy between CD103$^+$ and CD103$^-$ CD8$^+$CD69$^+$ Trm cells was not found in the epithelial layer. Itgb2high CD8$^+$CD69$^+$CD103$^-$ Trm cells constituted on average 8.9% of epithelial CD8$^+$ T cells, and KLRG1$^+$ CD8$^+$CD69$^+$CD103$^-$ Trm cells comprised 1.9% of CD8$^+$ T cells in the epithelium (**Figure 4E**).

Figure 4 *CD8$^+$CD69$^+$CD103$^-$ T cell characterization. (A) Representative flow dotplot of Itgb2high and CD103$^+$ expression within lamina propria CD8$^+$CD69$^+$ T cells. (B) Representative flow dotplot of Itgb2high and GzmK (left) and KLRG1 (right) coexpression within lamina propria CD8$^+$CD69$^+$CD103$^-$ T cells. (C) Quantification of GzmK and KLRG1 within Itgb2$^+$/highCD103-, Itgb2-/lowCD103$^+$, and Itgb2-/lowCD103$^-$ lamina propria CD8$^+$CD69$^+$ T cells for healthy control subjects (n = 6-7; circles), CD patients from inflamed (diamonds) and noninflamed (triangles) ileum (paired, n = 4-6). Bars represent median and interquartile range. Comparison was made with a 1-way analysis of variance. (D) Representative flow dotplot including Fluorescence Minus One (FMO) control and quantification of PD-1 within Itgb2highCD103$^-$ and Itgb2-/lowCD103$^+$ lamina propria CD8$^+$CD69$^+$ T cells. Symbols and n as per panel C. Bars represent median and interquartile range. Comparison was made with a paired 2-tailed t test. (E) Quantification of Itgb2high and KLRG1 within epithelial CD8$^+$CD69$^+$CD103$^-$ T cells. Symbols as per panel C. Healthy control subjects: n = 7; CD patients: n = 4. Bars represent median and interquartile range.*

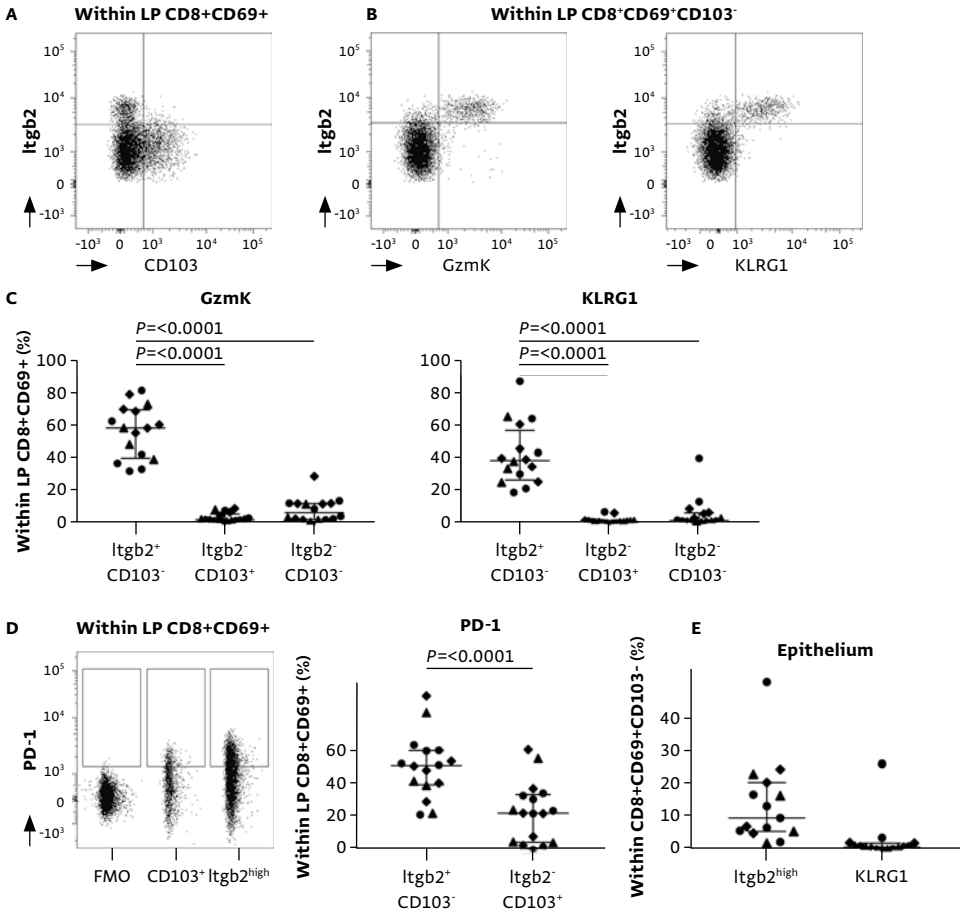
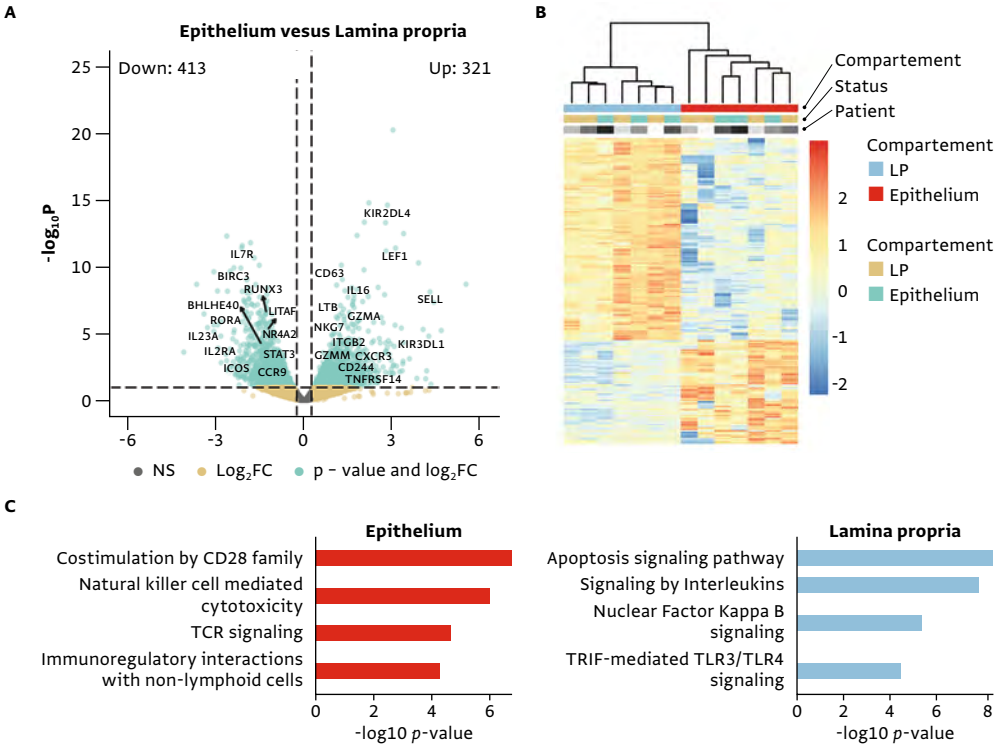


Figure 5 *Location shapes the profile of intestinal CD103⁺ CD69⁺CD8⁺ T cells. (A) Volcano plot of the expressed genes, with a nominal P value <0.99, comparing intraepithelial to lamina propria CD8⁺CD69⁺CD103⁺ T cells; selected genes are highlighted. On the x-axis the log2 fold change (log2FC) is shown, and on the y-axis the -log10 P value (-log10P) is shown. Grey indicates not significantly differentially expressed genes, yellow indicates genes with a log2FC >0.25 and -log10P > 10 × 10−2.5 , green indicates genes with a log2FC >0.25 and -log10P < 10 × 10−2.5. (B) Heatmap of the top 200 differentially expressed genes comparing CD103⁺ intraepithelial and lamina propria T cells with hierarchical clustering on the columns concerning compartment, status and patient. Rows are z score normalized. (C) Pathway terms related to the 321 genes upregulated in intraepithelial (top) and 413 genes upregulated in lamina propria (bottom) CD8⁺CD69⁺CD103⁺ T cells. (D) Messenger RNA (mRNA) expression (log2 counts; right) and percentage (left) of ileal CD8⁺CD69⁺CD103⁺ T cells expressing IL7R (CD127) in healthy control subjects (n = 3-5; diamonds) and CD patients from inflamed (circles) and noninflamed (triangles) ileum (paired, n = 4-5). Comparison was performed with Wald's statistic and a paired 2-tailed t test, respectively. (E) Gene set enrichment analysis of Trm genes in humans (identified by Hombrink et al25) in pairwise comparisons involving intraepithelial and lamina propria CD8⁺CD69⁺CD103⁺ T cells derived from the ileum of healthy adult control subjects and CD patients pooled, represented by the normalized enrichment score and FDR statistical value (FDRq). (F) Representative gating strategy including Fluorescence Minus One (FMO) control (upper panel) and quantification (lower panel) of CD63 in CD8⁺CD69⁺CD103⁺ T cells comparing epithelium (IE) and lamina propria (LP). Bars represent median and interquartile range. Comparison was performed with a paired 1-tailed t test. Symbols as per panel D. Healthy control subjects: n = 7, CD patients: n = 3-6. (G) As per panel F but for CXCR3. (H) As per panel F but for TIM-3 (left) and TIGIT (right). Comparison was performed with a paired 2-tailed t test.*



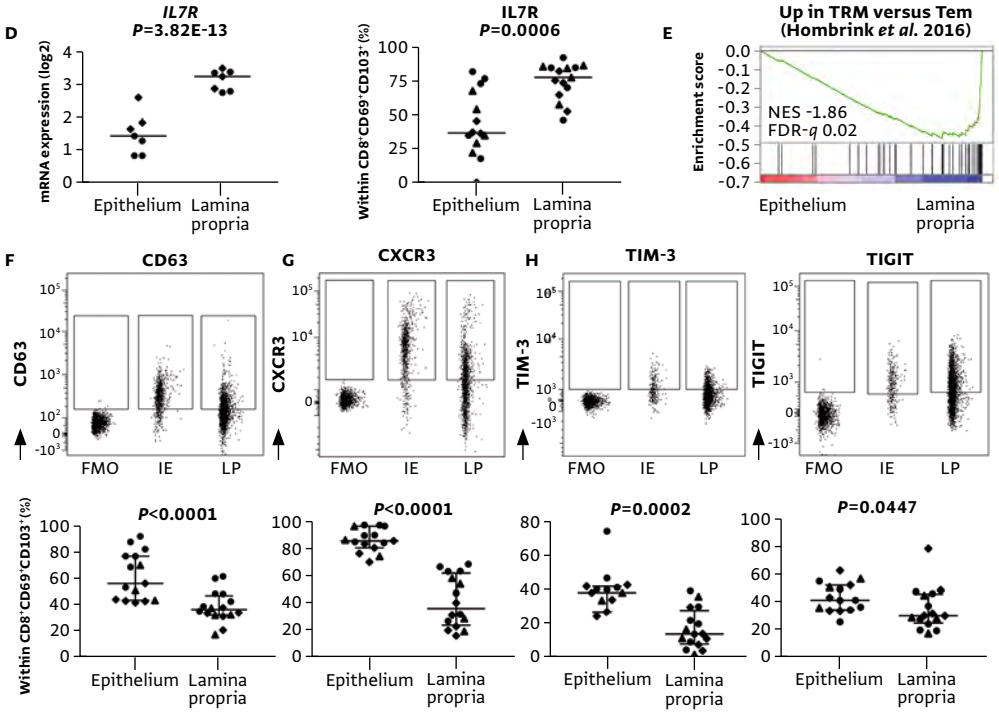
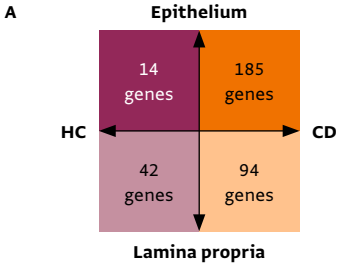
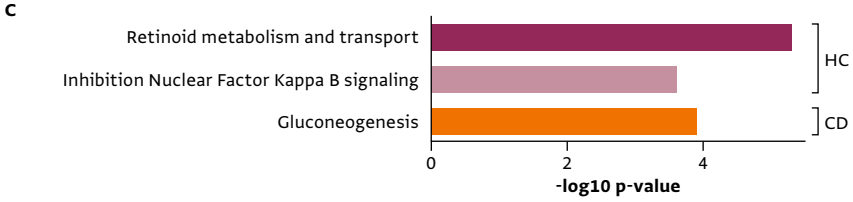


Figure 6 *During inflammation, most upregulated genes are found in CD8⁺ Trm from the epithelium. (A) Diagram showing the differentially expressed genes that are specific, ie not differentially expressed in the other disease state, for healthy control (HC) subjects (left) or CD patients (right). The upper part depicts the number of genes that are upregulated and the lower part those that are downregulated in intraepithelial compared with lamina propria CD8⁺CD69⁺CD103⁺ T cells from the ileum. (B) Selection of top genes up- and downregulated as per panel A. (C) Pathway terms related to the genes specific for CD8⁺ T cells from HC subjects or CD patients for both lamina propria and epithelium derived from panel A (colors reflect the colors of panel A).*



B

Epithelium		Lamina propria	
HC	**CD**	**HC**	**CD**
APOC3	CCL4L1	IFNG	BATF
DHRS4L1	CD37	IFRD1	CEACAM1
RBP2	IER2	ITGA4	FBXO33
	ITGB7	NFKBIB	GPR171
	KLF2	NFKBIE	GPR65
	MIF	RELT	KAT6b
	TELO2		LGALS3



The transcriptional profile of ileal CD8$^+$CD69$^+$CD103$^+$ T cells is largely dependent on mucosal localization

Next, we focused on the differences of CD8$^+$CD103$^+$ Trm cell profiles, based on the their spatial distribution. Differential gene expression of intraepithelial compared with lamina propria CD8$^+$CD69$^+$CD103$^+$ T cells revealed 321 upregulated and 413 downregulated genes shared by CD patients and healthy control subjects (FDR < 0.1) (**Figure 5A** and **B**; **Supplementary Table 1**). Subsequent pathway analysis showed enrichment of T cell receptor signaling, cytotoxicity, and interaction with nonimmune cells in the epithelial compartment (**Figure 5B**, left panel). In the lamina propria, cytokine signaling and inhibition of apoptosis were enriched (**Figure 5B**, right panel). In support of the latter, expression of interleukin (IL)-7R, which is essential for T cell homeostasis and long-term survival,[23,24] was upregulated at both messenger RNA and protein level in lamina propria CD8$^+$CD69$^+$CD103$^+$ T cells (**Figure 5D**). Furthermore, classical CD8$^+$ Trm cell genes, including *RUNX3*, *NR4A2*, *ICOS*, and *LITAF*, showed higher expression in lamina propria CD8$^+$CD69$^+$CD103$^+$ T cells (**Figure 5A**). The more profound Trm cell profile of lamina propria compared with intraepithelial CD8$^+$CD69$^+$CD103$^+$ T cells was also supported by gene set enrichment analyses for a core Trm cell gene set (**Figure 5E**).[25] However, intraepithelial CD8$^+$CD69$^+$CD103$^+$ T cells did not show enrichment of core effector memory or central memory T cell-related gene sets. We did observe elevated expression of cytotoxic genes such as *NKG7*, *GZMM*, *LTB*, *GZMA*, and killer-immunoglobulin receptors (*KIR2DL4*, *KIR3DL1*, *KIR2DS4*) (**Figure 5A**) in the epithelial subset. Even though KIRs were elevated on messenger RNA level, no difference for the inhibitory KIRs was observed on protein level, and intraepithelial CD8$^+$ T cell KIR expression was low overall (average KIR3DL1 expression of 3.8% in the epithelium). Elevated expression of CD63 was observed in intraepithelial compared with lamina propria CD8$^+$CD69$^+$CD103$^+$ T cells on protein level (**Figure 5F**), indicative of secretory vesicles containing cytotoxic proteins. Furthermore, CXCR3 was highly expressed on epithelial CD8$^+$CD69$^+$CD103$^+$ T cells (**Figure 5G**) supporting immunoregulatory interactions with nonlymphoid cells (**Figure 5C**, left panel), as its ligands are expressed by epithelial cells.[26] In addition, the immune checkpoints TIM-3 (HAVCR2) and TIGIT were more highly expressed by epithelial CD8$^+$CD69$^+$CD103$^+$ T cells (**Figure 5H**). In summary, CD8$^+$CD69$^+$CD103$^+$ T cells in the lamina propria show a more classical Trm cell profile and cytokine signaling, whereas in the epithelium a tightly regulated innate-like cytotoxic profile is more pronounced.

Different profiles in healthy control subjects and CD patients

Besides compartmental differences, an inflammatory milieu can influence the transcriptomic and functional profile of tissue T cells. Transcriptional differences between CD103$^+$ and CD103$^-$ CD8$^+$ Trm cells in the lamina propria induced by inflammation were minimal. Only *OASL* and *CCL4* were more highly expressed in CD103$^-$ compared with CD103$^+$ CD8$^+$ Trm cells in inflamed ileum of CD patients. Differences between transcrip-

tional profiles of $CD8^+CD69^+CD103^+$ T cells between healthy control subjects and active CD patients were mainly found in the epithelium (disease-specific genes: 185 genes in the CD epithelium, 94 genes in the CD lamina propria, and 14 and 42 genes for healthy control subjects, respectively) (**Figure 6A**; **Supplementary Table 1**). CD-specific genes included the innate proinflammatory *IER2* and *MIF* for intraepithelial $CD8^+CD69^+CD103^+$ T cells, and in the lamina propria, *BATF* and *LGALS3*, (encoding Galectin-3), both previously associated as drivers of IBD inflammation (**Figure 6B**).[27,28] On the pathway level, there was enrichment of gluconeogenesis in CD patients, whereas in healthy control subjects retinoid (vitamin A) metabolism was enriched in intraepithelial $CD8^+CD69^+CD103^+$ T cells (**Figure 4C**). Together this indicates that inflammation in CD patients primarily affects the profile of $CD8^+CD69^+CD103^+$ T cells in the epithelium.

Discussion

In the present study, we demonstrate that intestinal $CD8^+$ Trm cell transcription profiles depend on their mucosal localization. Lamina propria located $CD8^+CD103^+$ T cells have a classical Trm cell profile with active pathways for regulating longevity and cytokine signaling, while intraepithelial $CD8^+CD103^+$ T cells actively sense the external environment as part of the mucosal barrier and display enrichment in natural killer receptors and innate-like markers in line with previous studies in both mice and humans.[29,30] The changes seen during active inflammation are more pronounced in the intraepithelial $CD8^+CD103^+$ T cell subset, leading to an innate proinflammatory profile with a concurrent loss of homeostatic functions such as vitamin metabolism. These data support recent observations in ulcerative colitis in which disease-susceptibility loci were mostly enriched in intraepithelial $CD8^+$ T cells, especially during active inflammation.[8] Furthermore, the differences observed between epithelial and lamina propria $CD8^+CD103^+$ Trm cells suggest that potential infiltrating cells from the lamina propria in the epithelial layer also acquire a proinflammatory innate profile. The microenvironment thus has an important role in skewing a cell's phenotype.

We also describe transcriptomic and protein differences between CD103- and $CD103^+$ $CD8^+$ Trm cells in the lamina propria, which we corroborate and expand upon previous findings in the human intestine.[9,15,16] The $CD8^+CD103^-$ Trm cell subset in the lamina propria was defined by high Itgb2 expression further characterized by PD-1, GzmK, KLRG1 and EOMES. A recent study of donor-derived Trm cells after intestinal transplantation also described 2 transcriptionally different CD8 Trm cell subsets, where the $CD8^+CD69^+CD103^-$ subset characterized by coexpression of *ITGB2* displayed a more cytotoxic profile compared with the $CD8^+CD69^+CD103^+$ subset.[16] Similar findings were recently reported in a study of ileum samples obtained after ileocecal resection in CD patients demonstrating a statistically significant increase in percentages of $CD8^+CD103^-$

KLRG1$^+$ Trm cells in inflamed compared with noninflamed ileum and healthy control subjects, with no difference for the CD8$^+$CD103$^+$ subset.[15] The CD8$^+$CD103$^-$KLRG1$^+$ expressed higher levels of GZMB, whereas CD8$^+$CD103$^+$ Trm cells expressed higher levels of IL-22, IL-26 and CCL20.[15]

Our data show a decrease in CD8$^+$ T cells per µm2 in both the epithelium and lamina propria in inflamed ileum of CD patients compared with paired noninflamed ileum and the ileum of healthy control subjects. Additionally, within CD8$^+$CD69$^+$ T cells a decrease in CD103$^+$ Trm cells and a relative increase of CD103$^-$Itgb2highKLRG1$^+$GzmK$^+$ Trm cells was observed in inflamed ileum of CD patients. Recently, Tkachev et al31 observed that pathogenic cells in graft-vs-host disease in a simian transplantation model comprise rapidly developed CD8$^+$CD69$^+$ Trm cells, which were CD103$^-$ but expressed *ITGB2*, *CCL4L1*, *CD74* and *CCL3* among others. Another study recently linked appearance and accumulation of a GZMK$^+$ CD8$^+$ T cell population to an inflammatory phenotype in immune aging. This subset is characterized by both high PD-1 and TIGIT expression, is clonally expanded, and is regulated by EOMES and BATF.[22] These data suggest that within the CD103$^-$ CD8$^+$ Trm cell population in the lamina propria, a CD69$^+$CD103$^-$ Itgb2highGzmK$^+$KLRG1$^+$ Trm cell subset with pathogenic potential is present.

In line with our study, single cell RNA-sequencing of colonic T cells showed presence of multiple CD8$^+$ Trm cell clusters, of which a KLRG1$^+$EOMES$^+$ITGB2$^+$ subset is enriched in ulcerative colitis, and the CD103$^+$ population in healthy control subjects.[32] TCR analysis showed overlap between all CD8$^+$ Trm cell clusters except for between these distinct CD8$^+$ Trm cell subsets.[32] This was similar to findings for KLRG1$^+$CD103- and KLRG1-CD103$^+$ CD8$^+$ T cells in ileal transplant material.[9] These distinct CD8$^+$ Trm cell subsets thus seem to originate from different CD8$^+$CD69$^+$ T cells. CD103$^+$ CD8$^+$ Trm cells residing in the epithelium and the lamina propria, however, had similar TCR repertoires,9 indicating that they are derived from the same pool.

Lamina propria and epithelial differences, described in the present study, could be partially due to adhesion of CD103 (integrin αE) to E-cadherin, which initiates intracellular signaling to advance effector functions.[33] The receptor E-cadherin is only expressed in the epithelium, so CD103 expression in the lamina propria could be redundant and therefore exert less influence on the function of the cell. Further fine-tuning of the functional profile is most likely induced by local cues.[4,7] Whether, the severity of inflammation correlates with the magnitude of CD103$^+$CD8$^+$ T cell changes, both in number and in functional profile, is unknown.

Etrolizumab (anti-integrin β7) has been shown to be promising in phase II and III clinical trials in IBD.[34,35] In vitro, etrolizumab induces internalization of integrin β7, impairing its adhesion to MAdCAM-1, and blocking migration of immune cells to the gut, and has therefore a similar mode of action as the anti-integrin α4β7 antibody vedolizumab.[36] Additionally, etrolizumab affects the adhesion of integrin αE to E-cadherin result-

ing in decreasing intraepithelial $CD103^+$ cell counts (without distinction for immune cell type)34 and in a reduced accumulation of mainly $CD8^+$ and T helper 9 cells.[37] Thus, primarily the $CD8^+CD103^+$ Trm cell subset seems to be targeted by etrolizumab. The question is whether this is desirable because $CD4^+CD69^+CD103^+$ and not $CD8^+CD69^+CD103^+$ T cells have been correlated with disease flares in IBD.[11]

The fact that higher *ITGAE* (integrin αE/CD103) counts at baseline as reported in the phase II etrolizumab trial were related to higher therapeutic response rates does not underline a pathogenic role of the $CD8^+CD103^+$ subset, as this was demonstrated in bulk data34 and thus reflected a combination of dendritic cells, $CD4^+$ and/or $CD8^+$ T cells. Additionally, post hoc analysis of the latter study showed that patients with high *ITGAE* counts before treatment had milder disease activity with a lower endoscopic disease score at baseline.[13] This corresponds with our observation that patients with milder disease, defined as a lower simple endoscopic score for CD of the ileum, had higher mucosal levels of $CD8^+CD103^+$ T cells.

Our findings demonstrate the heterogeneity and dual functionality of Trm cell subsets in the intestinal mucosa. Long-term integrin β7 blockade could have a negative impact on the presence, and thus homeostatic functions, of these $CD8^+CD103^+$ T cells, which clearly warrants further evaluation. For example, $CD8^+$ $CD103^+$ T cells might contribute to vitamin A metabolism which is essential in maintaining epithelial integrity.[38] The suggestion that $CD8^+CD103^+$ T cells in IBD patients in remission regain a regulatory profile[14] should be further studied in a longitudinal cohort. Altogether, these differences indicate that therapeutic strategies could have a different impact on the same immune cells depending on the compartment of residence and presence of an inflammatory milieu, and should be taken into account when investigating short- and long-term effects of new gut T cell-targeting drugs. In conclusion, the transcriptional profile of $CD8^+$ Trm cells differs depending on the degree of inflammation and location within the gut.

Methods

Patient inclusion

Patients with CD, most newly diagnosed, were prospectively enrolled at the outpatient clinic of the Rijnstate Crohn and Colitis Centre (Arnhem, the Netherlands). During ileocolonoscopy multiple biopsy specimens were taken for histopathological analysis, for immunophenotyping by flow cytometry analysis (n = 27), and for RNA-sequencing of sorted subsets and imaging mass cytometry (n = 4). Healthy control subjects (n = 10) underwent ileocolonoscopy for polyp surveillance or iron deficiency. They had normal macroscopical ileal mucosa, which was confirmed by histology (see **Table 1** for patient characteristics).

Table 1 *Baseline patient characteristics.*

	Flow cytometric analysis		RNA-seq		CyTOF	
	CD Patients (n = 27)	**HC Subjects** (n = 7)	**CD Patients** (n = 4)	**HC Subjects** (n = 3)	**CD Patients** (n = 3)	**HC Subjects** (n=2)
Gender						
• Female	16 (76.2)	4 (57.1)	3 (75)	2 (66.7)	2 (66.7)	1 (50)
• Male	5 (23.8)	3 (42.8)	1 (25)	1 (33.3)	1 (33.3)	1 (50)
Age, y	24 (20-32)	50 (46-60)	49 (30-54)	36	46	36
Smoking status						
• Yes	11 (52.4)	0 (0)	2 (50)	2 (66.7)	2 (66.7)	2 (100)
• No	7 (33.3)	7 (100)	0 (0)	1 (33.3)	0 (0)	0 (0)
• Ceased	3 (14.3)	0 (0)	2 (50)	0 (0)	1 (33.3)	0 (0)
Duration of complaints before ileocolonoscopy, wk	14 (9-23)	NA	4 (1-6)	NA	4	NA
Calprotectin, µg/g	231 (156-487)	NA	120 (51-728)	NA	139	NA
CRP, mg/L	25 (11-62)	NA	9 (4-19)	NA	4	NA
Treatment at ileocolonoscopy						
• None	22 (81.4)	7 (100)	4 (100)	3 (100)	3 (100)	2 (100)
• 5-ASA	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
• Steroids	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
• Thiopurine	1 (4.5)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
• Mesalamine + thiopurine	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
• Anti-TNF	3 (13.6)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
• Anti-IL12/23	1 (4.5)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
HBI score						
• <5, remission	2 (7.4)	NA	1 (25)	NA	0 (0)	NA
• mild disease	12 (44.4)		2 (50)		2 (66.7)	
• moderate disease	9 (33.3)		1 (25)		1 (33.3)	
• >16 severe disease	4 (14.8)		0 (0)		0 (0)	
SES-CD score						
• inactive disease	0 (0)	NA	0 (0)	NA	0 (0)	NA
• mild disease	8 (29.6)		1 (25)		1 (33.3)	
• moderate disease	12 (44.4)		2 (50)		1 (33.3)	
• ≥16 severe disease	7 (25.9)		1 (25)		3 (33.3)	
Montreal CD						
• Location	10 (37)	NA	3 (75)	NA	2 (66.7)	NA
○ L1: ileal	0 (0)		0 (0)		0 (0)	
○ L2: colonic	17 (73)		1 (25)		1 (33.3)	
○ L3: ileocolonic						
• Behaviour						
○ B1: nonstricturing, nonpenetrating	23 (85.2)		1 (25)		1 (33.3)	
○ B2: stricturing	3 (11.1)		3 (75)		2 (66.7)	
○ B3: penetrating	1 (3.7)		0 (0)		0 (0)	

Values expressed in n (%) or as median with interquartile range.
CD, Crohn's disease; CRP, C-reactive protein; HBI, Harvey-Bradshaw index; HC, healthy control; RNA-seq, RNA-sequencing; SES-CD, simple endoscopic score for CD; TNF, tumor necrosis factor.

Mechanical cell isolation

Biopsies for analysis without separation of the lamina propria and epithelium were stored in a phosphate-buffered saline solution at 2-8°C, after which flow cytometric analysis was performed within 8 hours. We carried out mechanical preparation of single cell suspensions. Hereto, specimens were pooled and blended in Hank's Balanced Salt Solution (HBSS) (Gibco, Waltham, MA) supplemented with 1% bovine serum albumin (BSA) using a 70-mm gaze and spatula followed by Ficoll density gradient centrifugation. The homogenate was resuspended, after washing, in 0.5 mL HBSS/1% BSA.

Enzymatic cell isolation

Biopsies collected in HBSS media containing 2% fetal calf serum (FCS) and 0.2% amphotericin B. The intestinal tissue was transferred to HBSS supplemented with 1 mM DTT (Sigma-Aldrich, St Louis, MO) and placed on a rolling device for 10 minutes at 4°C. After discarding the supernatant, the intestinal tissue was transferred to HBSS supplemented with 2% FCS and 5 mM EDTA and shaken (2×) at 180 rpm for 30 minutes at 37°C. The tissue suspension was passed through a 70-µm cell strainer (Costar, Greiner Bio-One, Germany) and constituted the intraepithelial population. To obtain lamina propria T cells, intestinal biopsies were subsequently incubated for 1 hour at 37°C with 1 mg/ml Collagenase IV (Sigma-Aldrich) in RPMI medium (supplemented with 10% FCS, 100 U/mL penicillin-streptomycin, and 0.2% amphotericin B), then forcefully resuspended through a 19G needle, washed and filtered with 70-µm cell strainer (Costar). The cell suspensions were used for RNA-sequencing after sorting different T cell subsets.

Imaging mass cytometry

Intestinal biopsies were fixed in 10% neutral buffered formalin, paraffin-embedded, and 2 slides containing consecutive 4-µm-thick sections of all samples were prepared. One slide was stained with hematoxylin and eosin for histological assessment and the second slide was stained for IMC. IMC combines immunohistochemistry with high-resolution laser ablation of stained tissue sections followed by CyTOF mass cytometry enabling imaging of multiple proteins at subcellular resolution.[39] All antibodies were conjugated to lanthanide metals (Fluidigm, San Fransisco, CA) using the MaxPar antibody labeling kit and protocol (Fluidigm), and eluted in antibody stabilization buffer (Candor Bioscience, Wangen, Germany) for storage.

The slide was baked for 1.5 hours at 60°C, deparaffinized with fresh xylene for 20 minutes, and subsequently rehydrated in descending grades of ethanol (100% [10 minutes], 95%, 80%, 70% [5 minutes each]). After washing for 5 minutes in Milli-Q and 10

minutes in phosphate-buffered saline containing 0.1% Tween-20 (PBST), heat-induced epitope retrieval was conducted in Tris/EDTA (10 mM/1 mM, pH 9.5) for 30 minutes in a 95°C water bath. The slide was allowed to cool to 70°C before washing in PBST for 10 minutes. To decrease nonspecific antibody binding, tissue sections were blocked with 3% BSA and Human TruStain FcX (1:100, BioLegend, San Diego, CA) in PBST for 1 hour at room temperature. The antibody cocktail was prepared by mixing all antibodies at concentrations specific for the assay in PBST+0.5% BSA. After careful removal of the blocking buffer, the slide was incubated overnight at 4°C with the antibody cocktail. Antibodies used were E-cadherin 142Nd (metal tag) (clone 24E10, CST3195BF, Cell Signaling Technology, Danvers, MA), CD103 153Eu (clone EPR4166(2), ab221210, Abcam, Cambridge, United Kingdom), CD8α 162Dy (clone C8/144B, 14-0085-82, Thermo Fisher Scientific, Waltham, MA). Following three 5-minute washes in PBST and rinsing in Milli-Q, the tissue was counterstained with 0.1% toluidine blue for 5 minutes to enable tissue structure visualization under bright field microscopy if desired. Upon washing for 5 minutes in Milli-Q, the slide was incubated with Ir-intercalator (1:500 in PBST, Fluidigm) for 60 minutes at room temperature. Finally, the slide was washed in Milli-Q and air dried for at least for 20 minutes at room temperature.

Images were acquired at a resolution of 1 µm using a Hyperion Imaging System (Fluidigm). Regions of interest were selected based on the hematoxylin and eosin slides after which areas with an approximate size of 1000 × 1000 µm were ablated and acquired at 200 Hz. Pseudo-colored intensity maps were generated of each mass channel. Composite images were created and analyzed for each sample using ImageJ (version 1.47; National Institutes of Health, Bethesda, MD), and any changes to the brightness or contrast of a given marker were consistent across all samples.

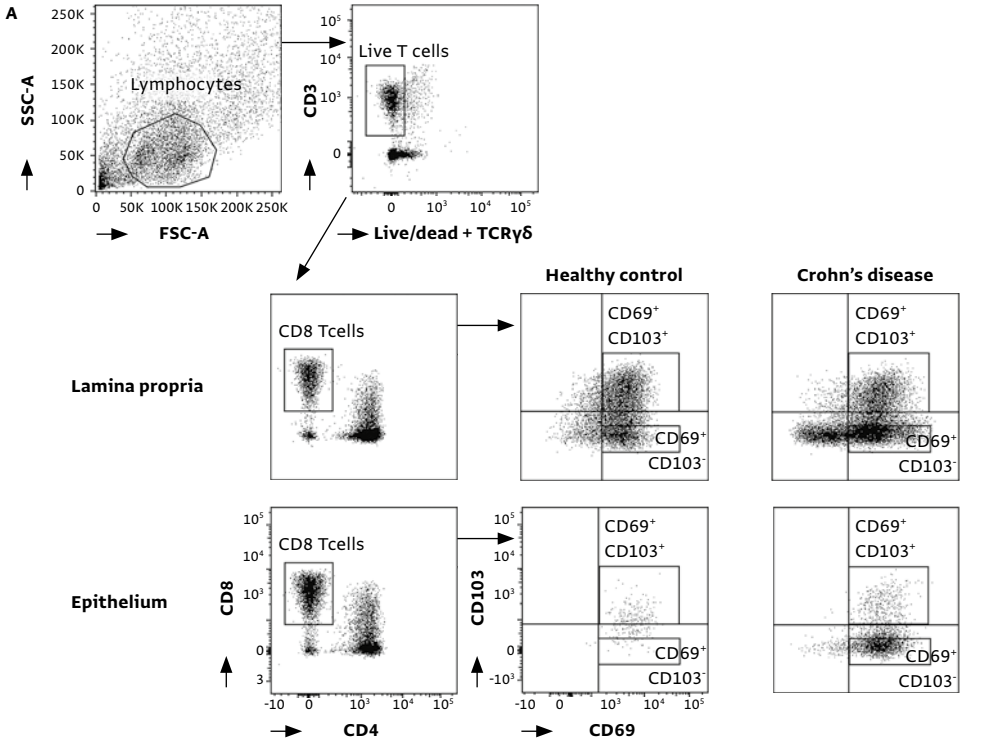
Flow cytometry

For flow cytometric analysis, the intestinal cells were incubated with surface antibodies for 20 minutes at 4°C. Antibodies used were fixable viability dye eF506 (65-2860-40; eBioscience, San Diego, CA), anti-human CD3 APC-H7 (clone SK7, 560176; BD Biosciences), CD8α PerCP-Cy5.5 (clone SK1, 565310; BD Biosciences), CD8α BV650 (clone UCHT1, 563822; BD Biosciences), CD69 PE (clone L78, 555531; BD Biosciences), CD69 PE-Cy7 (clone FN50, 557745; BD Biosciences), CD103 PE (clone Ber-ACT8, 550260; BD Biosciences), CD103 FITC (clone Ber-ACT8, 550259; BD Biosciences), TIM-3 BV711 (clone 7D3, 565566; BD Biosciences), PD-1 BV711 (clone EH12.1, 564017; BD Biosciences), Itgb2/CD18 FITC (clone TS1/18, 302105; Biolegend), CD3 BV605 (clone UCHT1, 300460; Biolegend), KLRG1 PE-CF594 (clone 14C2A07, 368608; Biolegend), CXCR3 BV605 (clone G025H7, 353728; Biolegend), CD3 AF700 (clone UCHT1, 300424; Biolegend), CD63 FITC (clone H5C6, 353006; Biolegend), CD4 BV785 (clone OKT4, 317442; Biolegend), and TCRγδ BV510 (clone B1, 331220; Biolegend), and TIGIT PerCP-eF710 (clone MBSA43, 46-9500-42; eBioscience). For intracellular staining cells were fixed and permeabilized using eBioscience

Fixation and Permeabilization buffers (Invitrogen, Waltham, MA) and stained with intracellular antibodies for 60 minutes at 4°C. Antibodies used were anti-human Ki-67 PE-Cy7 (clone B56, 561283; BD Biosciences), EOMES APC-eF780 (clone WD1928, 47-4877-42; eBioscience), KIR3DL1 BV421 (clone DX9, 312714; Biolegend), Granzyme K PerCP-Cy5.5 (clone GM26E7, 370514; Biolegend), and KIR2DL4 AF700 (clone 181703, FAB2238N-100UG; R&D Systems, Minneapolis, MN). Measurement was performed on a FACSCanto (BD Biosciences) or LSR Fortessa (BD Biosciences) (for gating strategy see **Figure 7**).

For sorting the intestinal cells were incubated with the surface antibodies for 20 minutes in supplemented RPMI (2% FCS, 1% penicillin and streptomycin, 0.2% amphotericin B) at 4°C, and subsequently washed in fluorescence activated cell sorting buffer before sorting on a FACSAria III (BD Biosciences) (for gating strategy see **Figure 7**). Antibodies used were fixable viability dye eF506 (65-2860-40; eBioscience), anti-human TCRγδ BV510 (clone B1, 331220; Biolegend), CD3 AF700 (clone UCHT1, 300424; Biolegend), CD4 BV785 (clone OKT4, 317442; Biolegend), CD8α APC-Cy7 (clone SK1, 557834;

Figure 7 *Gating strategy of intestinal CD103⁺ and CD103- CD69⁺CD8⁺ T cells. Gating for fluorescence-activated cell sorting for RNA-sequencing (strict gates) and flow cytometry (quadrant gates).*



BD Biosciences), CD127 BV421 (clone HIL-7R-M21, 562436; BD Biosciences), CD25 PE-Cy7 (clone M-A251, 557741; BD Biosciences), CD69 PE (clone FN50, 555531; BD Biosciences), CD103 FITC (clone 2G5, 550259; Beckman Coulter). Flow data was analyzed using FlowJo v10 (TreeStar, Ashland, OR).

RNA-sequencing

The sorted cells were thawed for TRIzol (Thermo Fisher Scientific) RNA extraction and stored at −80°C until library preparation. Sequencing libraries were prepared using the Cel-Seq2 Sample Preparation Protocol and sequenced as 75 bp paired-end on a NextSeq 500 (Utrecht Sequencing Facility). The reads were demultiplexed and aligned to the human complementary DNA reference genome (hg38) using BWA (version 0.7.13; http://bio-bwa.sourceforge.net/). Multiple reads mapping to the same gene with the same unique molecular identifier (6 bp long) were counted as a single read.

Raw counts of splice variants were summed and the raw counts were subsequently transformed employing variance stabilizing transformation. Ensembl names were converted to HGNC symbol, and if no symbol has been assigned the ensembl reference name was used. Differential analysis was performed using DESeq2 (Wald's test). For visualization purposes the R packages DESeq2, EnhancedVolcano and pheatmap were employed (R Foundation for Statistical Computing, Vienna, Austria). Raw counts were used as input for generating volcanoplots with the genes colored based on *P* value and log2 fold-change cutoffs, with selected gene symbols shown for the genes with an FDR < 0.1. For heatmaps, transformed counts were *z* score normalized followed by hierarchical clustering based on samples and genes. Pathway analysis was performed on the differentially expressed genes as input in Toppfun with standard settings. Gene set enrichment analysis , with as input the normalized data (output DESeq2), was used to assess enrichment of gene sets derived from the MSigDB C7 database (immunological signatures) and the Trm cell signature for human $CD8^+CD69^+CD103^+$ T cells as defined by Hombrink et al25. One thousand random permutations of the phenotypic subgroups was used to establish a null distribution of enrichment score against which a normalized enrichment score and FDR-corrected *q* values were calculated. RNA-sequencing data are available at GEO Accession GSE160925.

Statistical analyses

Flow cytometric data was analyzed with the independent 1-tailed or 2-tailed (paired) *t* test, or with a 1-way analysis of variance with post hoc Tukey's. For correlation analysis, Spearman's correlation was used. Data were analyzed with SPSS Statistics version 22.0.0.0 (IBM, Armonk, NY) and GraphPad Prism version 7.0 (GraphPad Software, San Diego, CA).

Ethics approval

The study protocol (NL28761.091.09 and TCBio 17/443, 17/444, 18/522) were approved by the research ethics committee of the Radboud University Nijmegen Medical Centre (CMO Regio Arnhem-Nijmegen, Nijmegen, the Netherlands) and the University Medical Center Utrecht, respectively. Written informed consent was obtained from each participating patient before any study-related procedure was performed. The procedures were performed in accordance with the Declaration of Helsinki.

All authors had access to the study data and reviewed and approved the final manuscript.

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CHAPTER 6A

AlphaE expression in IBD: a biomarker for the use of Etrolizumab?

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Britt Roosenboom

Ellen G. van Lochem

Carmen S. Horjus Talabur Horje

Dear editor

We congratulate Ichikawa and colleagues with their interesting paper on the alphaE integrin expression in the ileum and colon of inflammatory bowel disease (IBD) patients and healthy controls (HC).[1] AlphaEbeta7 is a transmembrane molecule on the surface of different mucosal immune cells, such as T cells, dendritic cells, innate lymphoid cells and plays a role in the retention of these cells in the mucosa by binding to E-cadherin on epithelial cells. The alphaEbeta7(αEβ7)$^+$ T cells are still being investigated for their role, either pro- or anti-inflammatory, in the pathogenesis of IBD.

Ichikawa et al suggest that αE levels can be used as a biomarker in selecting patients suitable for Etrolizumab treatment, based on their potentially pro-inflammatory role, higher levels in the ileum compared to colon and stability of these cells regardless of inflammation and medication use. Hereto they also cited findings from an earlier study on the relation between 'αEhigh' and 'αElow' status and remission rates on Etrolizumab treatment in ulcerative colitis (UC) patients.[2]

The argument used for the pro-inflammatory capacity of αE$^+$cells in IBD is based on the in vitro findings of increased secretion of pro-inflammatory cytokines by a subset of CD4$^+$αE$^+$cells.[3] However, this subset represents only a minority of all αE$^+$ T cells in UC patients; the majority of αEβ7 is expressed on CD8$^+$ tissue resident memory cells with an immunosurveillance and protective function.[4] There is no evidence that blocking the binding of αE$^+$cells to E-cadherin, will target the function of the intestinal T lymphocytes. Additionally, Zundler et al demonstrated that αE blockade does not reduce the accumulation of CD4$^+$αE$^+$T cells in the gut of IBD patients.[5] Up till now, the exact role for αE levels in the pathophysiology of IBD is not elucidated.

Considering the posthoc analysis following the Etrolizumab phase 2 trial, statistically significant histological improvement following treatment was only seen in UC patients with 'αElow' gene expression at baseline and not in 'αEhigh' patients.[2] Additionally, 'αEhigh' patients presented with statistically significant lower endoscopic and histological scores compared to 'αElow' patients before treatment2, which does not support the suggested pro-inflammatory role reserved for αE$^+$ cells in these patients. Finally, Ichikawa et al demonstrate no difference in αE$^+$levels between HC and IBD patients, nor any correlation with clinical, endoscopic or histological disease activity, questioning the use of αE levels as a biomarker in IBD.

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CHAPTER 6B

Analysis of intestinal T-cell subsets in inflammatory bowel disease

INFLAMMATORY BOWEL DISEASE 2020 JAN 6;26(2):E13

Britt Roosenboom

Ellen G. van Lochem

Carolijn Smids

Marcel J.M. Groenen

Peter J. Wahab

Carmen S. Horjus Talabur Horje

To the editors

We thank Escudero-Hernandez *et al.* for their interest in our paper investigating the frequency of CD103(αE)$^+$ T-cell subsets in colonic and ileal biopsies of patients with inflammatory bowel disease (IBD).[1] We demonstrated that CD103$^+$CD4$^+$ and CD103$^+$CD8$^+$ T-cell subsets represent only a minority of the T-cell infiltrate in inflamed biopsies, where the majority of mucosal T cells consists of the CD103$^-$CD4$^+$ cells.[1]

Escudero-Hernandez et al. investigated the frequency of CD45$^+$ cells and the proportion of CD3$^+$ cells within the CD45$^+$ cells in the epithelium of inflamed colon and ileum of patients with ulcerative colitis (UC), Crohn's disease (CD), healthy controls (HC) and non-IBD inflammation. They describe similar percentages of intraepithelial CD45$^+$ cells in CD and UC, next to an increased intraepithelial CD3$^+$/CD45$^+$ ratio in UC compared to CD and HC. This difference in the intraepithelial CD3$^+$/CD45$^+$ ratio, between UC and CD and non-IBD inflammation should be further investigated in a larger cohort, in order to determine its specificity for differentiating between different types of colitis.

Comparing the study of Escudero-Hernandez et al. with our studies on T cells in IBD is not relevant as the purpose of both studies is different.[1,2] Focusing on the intraepithelial lymphocytes indeed can be informative for the pathophysiology in IBD and possibly for discrimination between UC and CD. Both in the study of Smids et al., where we studied mucosal T-cell subpopulations as biomarker for disease activity and progression, as well as in our most recent study (Roosenboom et al.), there was no reason for separating epithelium from lamina propria.

In the present study we investigated different CD103$^+$ T-cell subsets, on whole biopsies, knowing that CD103 is presented on approximately 90% of intraepithelial T cells and 40% of T cells in the lamina propria and is a potential target in IBD as it can form a heterodimeric with β7 (anti-β7 integrin, Etrolizumab).[3,4] In order to study the possible role of CD103$^+$ T cells in the pathophysiology of IBD, it would be interesting to analyze these cells in the epithelium and lamina propria separately. However, investigation of whole biopsies would benefit the study of mucosal biomarkers, like CD103$^+$ T cells for selecting groups of patients suitable for treatment with Etrolizumab, in daily practice.

References

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CHAPTER 7

Distribution of mucosal PD-1 expressing T cells in patients with colitis of different etiologies

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Britt Roosenboom

Carmen S. Horjus Talabur Horje

Carolijn Smids

Jan Willem Leeuwis

Elly van Koolwijk

Marcel J.M. Groenen

Peter J. Wahab

Ellen G. van Lochem

Abstract

BACKGROUND Immunotherapy, targeting programmed death-1 (PD-1) enhances anti-tumor T-cell activity in patients with malignancies. Blocking PD-1 or its ligand may lead to fulminant colitis as serious adverse event in these patients. Since little is known of the presence and role of PD-1$^+$T cells in colitis of different etiologies, we determined PD-1$^+$T cells in mucosal specimens of patients with inflammatory bowel disease (IBD), infectious colitis (InfC), immunotherapy-related colitis (ImC) and healthy controls (HC).

METHODS Newly diagnosed patients with ulcerative colitis (UC, n=73), Crohn's disease (CD, n=50), InfC (n=5), ImC (n=8) and HC (n=8) were included. Baseline inflamed colonic biopsies were studied with immunohistochemistry and flowcytometry.

RESULTS Using immunohistochemistry, PD-1 was not present on lymphocytes in the epithelium of all patients, nor in HC. The percentage PD-1$^+$ of all lymphocytes in the lamina propria was 40% in UC, 5% in InfC, 3% in ImC and 0% in HC. Flowcytometry showed significant higher percentages of PD-1$^+$T cells in inflamed biopsy specimens of UC patients (22%) compared to all other groups: CD patients (13%), InfC (12%), ImC (5%) and HC (6%).

CONCLUSION There are relevant differences in distribution and frequencies of mucosal PD-1$^+$ T-cell subsets in patients with UC, CD, InfC and ImC, supporting the hypothesis that these types of colitis are driven by different immunological pathways. The increased numbers of PD-1$^+$ and PD-L1$^+$ lymphocytes in the colonic mucosa of UC patients suggest that the PD-1/PD-L1 pathway might be more activated in UC than in CD.

Introduction

Interaction of programmed death-1 (PD-1) with its ligand PD-L1 and/or PD-L2 controls several checkpoints that are crucial for the maintenance of immunologic tolerance in the gastrointestinal tract and prevent autoimmunity.[1] PD-1 is a co-inhibitory receptor expressed on B cells, T cells, monocytes and natural killer cells.[2] PD-L1 is expressed on antigen presenting cells, activated T cells and on various organs.[3] Whereas PD-1 activation suppresses T cells primarily in peripheral tissues, cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4, another checkpoint inhibitor regulating T-cell function) regulates T-cell proliferation especially in lymph nodes.[4]

Immune checkpoint inhibitors targeting PD-1 (nivolumab, pembrolizumab and cemiplimab) or PD-L1 (atezolizumab, durvalumab and avelumab) recently expanded the therapeutic oncological arsenal. They are approved in the treatment of non-small cell lung carcinoma (NSCLC), melanoma, hepatocellular carcinoma (HCC) and several other forms of cancer.[5] The PD-1/PD-L1 blockade results in the enhancement of lymphocyte activity, especially cytotoxic $CD8^+$ T cells, and reduction in numbers and suppressive activity of (intratumoral) Treg cells, in order to generate an antitumor immune response.[6,7]

Disturbance of the balance in the PD-1/PD-L1 system can lead to unwanted gastrointestinal adverse reactions, marking the crucial role of the PD-1 pathway in maintaining self-tolerance. Diarrhoea is found in 11% of patients treated with anti-PD-1 therapy, whereas immunotherapy-related colitis (ImC) confirmed with colonoscopy is described in 1.3% of patients treated with anti-PD-1 therapy and in 13.6% of patients treated with a combination of anti-PD-1 and anti-CTLA-4 (Ipilimumab).[8] Patients with mild PD-1 induced colitis displayed stable or slowly escalating symptoms over a period of months resembling clinical, endoscopic and histological features seen in patients with inflammatory bowel diseases (IBD), whereas CTLA-4 induced colitis shows a rapid progression resembling the course of infectious colitis (InfC).

ImC due to anti-PD-1 therapy is potentially severe, however it escalates more slowly than CTLA-4 induced colitis, possibly due to compensation by other regulatory mechanisms preventing complete loss of mucosal tolerance.[9,10]

Endoscopic similarities between ImC and ulcerative colitis (UC) include erythema of the gut mucosa, luminal bleeding, erosions and ulcerations with a continuous pattern of inflammation without complications such as fistulas.[11] Most histopathological features in UC and ImC are similar including the occurrence of cryptitis, ulcerations and crypt abscesses, but basal plasmacytosis and crypt distortion were less frequent in ImC, reflecting a more acute nature of the inflammatory proces opposed to the longer duration of symptoms before IBD is diagnosed.[12,13] In patients who discontinue treatment with anti-PD-1 antibodies, recurrence of colitis has been observed up to many months after treatment.[13] In these patients with recurrent colitis, histological features of chronicity (comparable to inflamed mucosa of IBD patients) were revealed.

The endoscopic and histological similarities between IBD and ImC raise the question to what extent defects in the PD-1/PD-L1 pathway contribute to the pathogenesis of IBD. Furthermore, inducing signaling through these receptors with agonist antibodies could potentially drive the immune system to a state of tolerance and thereby providing a new treatment target for IBD patients.[14]

Literature concerning the presence and immunological function of PD-1/PD-L1^{+} T cells in colitis of different etiologies is limited in humans and contradictory in mice 15,16,18,21, while phase I trials testing PD-1 receptor agonists in autoimmune diseases are underway.[22] The aim of the present study is to investigate the localization and numbers of PD-1 and PD-L1 expressing T cells in colonic mucosa biopsies of patients with InfC, ImC, newly diagnosed IBD patients and HC. Despite the similarities in clinical and histopathological presentation we hypothesize that UC, CD, InfC and ImC rely on different immunological mechanisms.

Patients and methods

Study population

Subjects were prospectively recruited between 2017 and 2019 at the Department of Gastroenterology in the Rijnstate hospital in Arnhem, The Netherlands. Patients, not previously known with IBD and suspected of UC or Crohn's disease (CD) based on clinical symptoms (chronic diarrhoea, rectal blood loss, abdominal pain or weight loss) underwent ileocolonoscopy. Endoscopic and histopathological indications corroborated diagnosis. Exclusion criteria consisted of concomitant or previous use of immunosuppressive medication or the presence of other autoimmune disorders. The Mayo score for UC patients and the SES-CD score for CD patients were used to assess endoscopic severity of disease activity at initial diagnosis.[23] The Montreal classification was used to classify UC and CD based on disease location and behaviour.[23]

Patients with histopathological analysis of biopsies and stool cultures proving the presence of bacteria (e.g. Campylobacter species or Shigatoxin-producing E. coli), were included in the group of patients with InfC.

Patients treated with immune checkpoint inhibitors (ICIs) blocking PD-1 in case of a diagnosed malignancy (e.g. NSCLC, renal cell carcinoma or Merkel cell carcinoma) experiencing complaints of diarrhoea and abdominal pain suspicious for ImC, underwent sigmoidoscopy or colonoscopy. They were included in this study when endoscopic and histopathological changes were observed, matching findings seen in ImC.[13] The following ImC-specific variables were extracted from the medical records at baseline endoscopy: received immunotherapy (e.g. pemprolizumab or nivolumab), indication of prescribed immunotherapy, the number of days from the last received immunotherapy infusion to endoscopy, the NCI-CTCAE (National Cancer Institute-Common Terminol-

ogy Criteria for Adverse Events: the grading scale provided for different adverse events) grade and the Mayo score in order to classify endoscopic severity.[24]

Healthy controls underwent ileocolonoscopy for polyp surveillance or iron deficiency. They did not present endoscopic abnormalities reflecting inflammation.

The variables age, gender and smoking were extracted from medical records.

Immunohistochemistry

First, immunohistochemistry (IHC) was performed on biopsied specimens of colonic mucosa of patients with UC, InfC, ImC and healthy controls to study the distribution of PD-1$^+$ lymphocytes. Given limited financial resources we were not able to perform IHC on the biopsies of the whole study population. Therefore, we chose to perform IHC in UC patients and not in CD patients, since inflammation of the mucosa is more uniform and continuous in UC compared to the very heterogeneous pattern in CD. The distribution of PD-1$^+$ lymphocytes in the colonic biopsies was analysed for a subgroup of the total number of included patients, to see whether biopsies for flowcytometry could be evaluated without separating lamina propria from the epithelium.

Biopsies were fixed in 10% neutral buffered formaline for 6-12 hours. Hereafter they were washed, dehydrated, embedded in paraffin, cut into 3-µm-thick sections and selected for immunohistochemistry. The 3-µm-thick sections were mounted on glass microscope slides. Besides hematoxylin and eosin (HE, from Klinipath) staining, samples were stained with the following monoclonal antibodies: CD3 (from Novocastra, clone LN10, 1:50, marking T cells), PD-1 (from Cell Marque, clone NAT105, concentration 3.28 µg/ml, marking the protein programmed death-1) and PD-L1 (from Ventana Roche, clone SP263, concentration 1.61µg/ml, marking the protein programmed death-ligand 1). A positive and negative tissue control for each staining run was performed on prequalified tonsil tissue. Matched patient tissue was stained with negative reagent control to assess nonspecific background staining. Slides were incubated with these antibodies in an automatic immunostainer (Ventana Benchmark Ultra, Eindhoven, the Netherlands). After performing immunostaining, these slides were scanned with an Intellisite high-resolution scanner (Philips ultra-fast scanner 1.6 RA; Philips Digital Pathology, Best, The Netherlands) and analyzed within the IntelliSite Pathology Solution Image Management System (IMS, Philips Digital Pathology, Best, The Netherlands).

The most affected biopsy at each HE stained slide was selected using the Geboes score.[25] The histological activity of disease was equal among the included patients with colitis for IHC (all with severe diffuse architectural changes (Geboes grade 0.3), marked increase of chronic inflammatory infiltrate (Geboes grade 1.3), moderate increase of neutrophils and eosinophils in the lamina propria (Geboes grade 2A.2 and 2B.2), less than 50% crypts with neutrophils in the epithelium (Geboes grade 3.2), marked crypt destruction (Geboes grade 4.2) and probable erosion-focally stripped (Geboes grade 5.2).

These stained biopsies were scored expressing the proportion of lymphocytes in the lamina propria surface and epithelial surface that were expressing PD-L1 and

PD-1, of any intensity (in percentages) at 40-fold magnification. Next to this percentage of positively stained cells, we scored the staining intensity as follows: 0 (negative), 1 (weak), 2 (moderate) and 3 (strong).

Flowcytometry

Four to six biopsied specimens taken from the inflamed parts of colonic mucosa of patients with UC, CD, InfC, ImC and non-inflamed colonic mucosa of HC and the same number of ileal biopsies of patients with CD and healthy controles were collected. Here we used biopsies of CD patients as a validation cohort for comparison with UC. When ulcerations were present, the biopsies were taken out of edges of the ulcerations. These biopsies were stored in a phosphate-buffered saline solution at 2-8 degrees, after which flowcytometric analysis was performed within 8 hours. We carried out mechanical preparation of single cell suspensions. Hereto, specimens were pooled and blended in Hanks'/1% bovine serum albumin using a 70-mm gaze and spatula followed by Ficoll density gradient centrifugation. The homogenate was resuspended, after washing, in 0.5 mL Hanks'/1% bovine serum albumin. The concentration of mononuclear cells in the suspension was estimated by microscopic counting with a KOVA glasstic slide (Hycor Biomedical Ltd., Penicuik, United Kingdom). Two hundred µL of the total cell suspension was used for flowcytometric analysis (FACSCanto, BD BiosciencesTM). Percentages of $CD3^+$ from the whole lymphocyte population, $CD4^+$, $CD8^+$ and PD-1^+ within $CD3^+$ T cells and $FoxP3^+CD25^+$ as a percentage of $CD3^+CD4^+$ T cells were reported. $CD4^+$, $CD8^+$, $CD45Ra^+$, $CD45R0^+$ and $CD103^+$ were also expressed as a percentage of the $CD3^+PD1^+$ subset. Antibodies and reagens used for flowcytometry were all obtained from Becton Dickinson Biosciences USA (**Supplementary Table 1**).

Statistical analysis

All variables were tested for normality using the Shapiro-Wilk test. In case of categorical data, variables were expressed as a number with percentage (%), where in case of continuous variables they were expressed as median with interquartile range (IQR). The Mann-Whitney-U test was used for the comparison between continuous variables of two independent groups, whereas the Kruskal-Wallis test was used for multiple independent groups. A two-sided p-value of 0.05 was considered to be statistically significant. Data analysis was performed using the SPSS statistical software (version 24.0.0.0; IBM Corp, Armonk, NY, USA) and GraphPad Prism (Graphpad Software version 7.0, La Jolla, CA, USA).

Ethics

The study protocol (NL28761.091.09) was approved by the research ethics committee of the Radboud University Nijmegen Medical Centre (CMO Regio Arnhem-Nijmegen, Nijmegen, The Netherlands). The procedures were performed in accordance with the Declaration of Helsinki (version 9, 19 October 2013).

Results

Patient characteristics

Baseline characteristics of all patients and HC are presented in **Table 1**. In total, biopsy specimens of 73 UC patients, 50 CD patients, 5 patients with InfC, 8 patients with ImC and 8 HC were included. Patients with UC, CD and InfC were younger than patients with ImC (33 years (IQR 24-45) versus 65 years (IQR 55-71)). Patients with UC, CD or InfC did not use immunosuppressive medication at time of ileocolonoscopy.

Table 1 *Baseline patient characteristics.*

	HC [n=8]	UC [n=73]	CD [n=50]	InfC [n=5]	ImC [n=8]
Gender					
• Female	6 (75)	38 (52)	29 (58)	2 (40)	2 (25)
• Male	2 (25)	35 (48)	21 (42)	3 (60)	6 (75)
Age	31 (26-37)	33 (26-45)	29 (22-41)	29 (21-57)	65 (55-71)
Smoking status					
• Yes	0 (0)	10 (13.7)	21 (42)	0 (0)	1 (12.5)
• No	4 (50)	42 (57.5)	23 (46)	5 (100)	3 (37.5)
• Ceased	4 (50)	18 (24.7)	6 (12)	0 (0)	4 (50)
• Unknown	0 (0)	3 (4.1)	0 (0)	0 (0)	0 (0)
Immunotherapy at baseline endoscopy,					
• Pembrolizumab	None	None	None	None	5 (62.5)
• Nivolumab					3 (37.5)
Indication immunotherapy					
• NSCLC	NA	NA	NA	NA	6 (75)
• Renal cell carcinoma					1 (12.5)
• Merkel cell carcinoma					1 (12.5)
Number of days from last received immunotherapy to colonoscopy	NA	NA	NA	NA	17 (11-32)
Pathologic bacteria causing colitis					
• STEC	None	None	None	3 (60)	None
• Campylobacter				2 (40)	
Mayo score					
• Mayo 0	NA	0 (0)	NA	NA	0 (0)
• Mayo 1		27 (37)			7 (87.5)
• Mayo 2		38 (52)			1 (12.5)
• Mayo 3		7 (10)			0 (0)
Montreal UC					
• Location	NA	25 (34)	NA	NA	NA
• E1: Ulcerative proctitis		24 (34)			
• E2: Left-sided UC		24 (34)			
• E3: Extensive UC					

SES-CD score					
• 0-2 inactive disease	NA	NA	0 (0)	NA	NA
• 3-6 mild disease			14 (28)		
• 7-15 moderate disease			20 (40)		
• ≥16 severe disease			16 (32)		
Montreal CD					
• Location	NA	NA	11 (22)	NA	NA
○ L1: ileal			14 (28)		
○ L2: colonic			25 (50)		
○ L3: ilealcolonic			40 (80)		
• Behaviour			6 (12)		
○ B1: non-stricturing, non-penetrating			3 (6)		
○ B2: stricturing					
○ B3: penetrating					
CTCAE scale					
• Grade 1	NA	NA	NA	NA	0 (0)
• Grade 2					7 (87.5)
• Grade 3					1 (12.5)
• Grade 4					0 (0)
• Grade 5					0 (0)

Values expressed in n (%) or as median with interquartile range; CD, Crohn's disease; CTCAE, Common Terminology Criteria for Adverse Events; HC, Healthy control; NA, Not Applicable; NSCLC, Non-small cell lung carcinoma; ImC, Immunotherapy-related colitis; InfC, Infectious colitis; UC, ulcerative colitis.

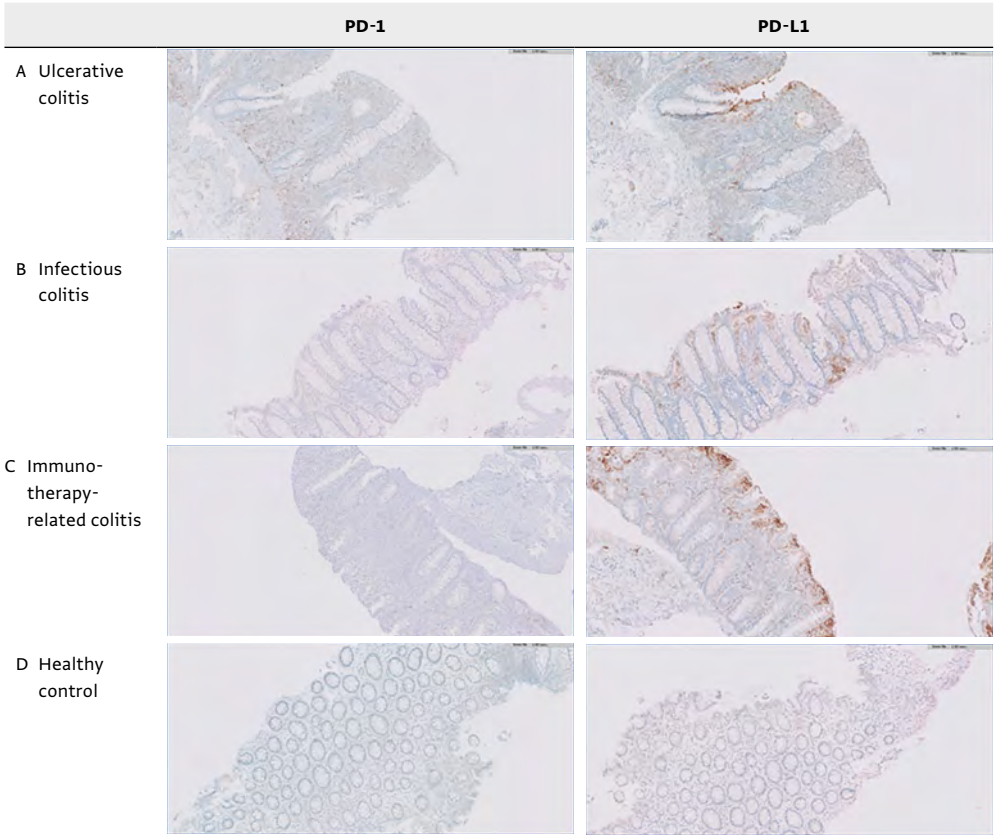
Three of five patients with InfC had a Shigatoxin-producing E. coli in their stool cultures, two had Campylobacter species. NSCLC was in most cases the underlying malignant disease wherefore patients received immunotherapy (75%). Five patients with ImC used pembrolizumab (62.5%) and three nivolumab (37.5%). The median time period between the last dosis immunotherapy and ileocolonoscopy was seventeen days (IQR 11-32).

Endoscopic disease severity, categorised as mild, moderate or severe disease, was evenly distributed in both UC and CD. However, in ImC there were more patients with milder disease activity according to the NCI-CTCAE scale.

Localization of PD-1 and PD-L1 in colitis

Immunostaining for PD-1 and PD-L1 was performed on formalin fixed, paraffin embedded biopsied colonic specimens of a subgroup of the total number included patients with equal histopathological severity of disease;10 UC patients, 4 patients with InfC, 4 patients with ImC and 4 HC (**Figure 1** and **Table 2**). PD-1 was not present on lymphocytes in the epithelial layer of patients with any type of colitis, nor in HC. Of all lymphocytes in the lamina propria, the highest frequency of PD-1^{+} lymphocytes was seen in UC patients (40% (9-63)), compared to InfC (5% (0-5)), ImC (3% (0-13)) and HC (0% (0-0)).

Figure 1 *Immunohistochemical staining indicating the presence of PD-1$^+$ and PD-L1$^+$ lymphocytes in colonic tissue of newly diagnosed patients with ulcerative colitis (A), infectious colitis (B), immunotherapy-related colitis (C) and in healthy controls (D). Representative photomicrographs, with a 20-fold magnification. PD-1, programmed death-1; PD-L1, programmed death-ligand1.*



In contrast to PD-1, the expression of PD-L1 on lymphocytes in the epithelial layer of UC and InfC was demonstrated with a mild to moderate staining intensity, but almost no epithelial PD-L1 was seen in HC and ImC. In all patients, the intensity of PD-L1 immunostaining of lymphocytes in the lamina propria was moderate to strong, with higher percentages compared to HC (UC 40% (20-63); InfC 30% (15-40); ImC 15% (11-19); HC 10% (5-14)).

Flowcytometric frequencies of T-cell subsets in colonic biopsies in colitis of different etiologies at diagnosis

The percentages of intestinal PD-1$^+$ T-cell subsets in colitis of different etiologies are displayed in **Table 3**, **Figure 2** and **Figure 3**.

Table 2 *The intensity of staining and proportion of PD-1 and PD-L1 in the lamina propria and epithelial layer of the colonic mucosa of patients with ulcerative colitis, infectious colitis, immunotherapy-related colitis and healthy controls.*

Immunohistochemistry Colon	HC n=4	UC n=10	InfC n=4	ImC n=4
PD-1 in lamina propria				
• % PD-1$^+$ lymphocytes	0 (0-0)	40 (9-63)	5 (0-5)	3 (0-13)
• Intensity of PD-1 (range 1-3)	0	1	1	1
PD-1 in the epithelial layer				
• % PD-1$^+$ lymphocytes	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)
• Intensity of PD-1 (range 1-3)	0	0	0	0
PD-L1 in lamina propria				
• % PD-L1$^+$ lymphocytes	10 (5-14)	40 (20-63)	30 (15-40)	15 (11-19)
• Intensity of PD-L1 (range 1-3)	1	2	2	3
PD-L1 in the epithelial layer				
• % PD-L1$^+$ lymphocytes	0 (0-0)	12.5 (9-20)	1 (0-5)	0 (0-4)
• Intensity of PD-L1 (range 1-3)	0	2	1	0

Values expressed in median percentages with interquartile range; Intensity of staining ranging from 1 to 3; HC, Healthy control; ImC, Immunotherapy-related colitis; InfC, infectious colitis; PD-1, programmed death-1; PD-L1, programmed death-ligand1; UC, ulcerative colitis.

Table 3 *Baseline percentages of CD4$^+$, CD8$^+$ and PD-1$^+$ T cells within CD3$^+$ T lymphocytes, subsets of PD-1$^+$ T cells (as % of PD-1$^+$ T cells) and FoxP3$^+$CD25$^+$ as percentage of CD3$^+$CD4$^+$ T cells explored with flowcytometry in colonic biopsies of healthy controls, patients with ulcerative colitis, Crohn's disease, infectious colitis and immunotherapy-related colitis.*

Flowcytometry Colon	HC n=8	UC n=73	CD n=50	InfC n=5	ImC n=8	p-value
CD4$^+$ as % of T cells	45 [32-72]	74 [65-81]	74 [66-80]	71 [47-79]	61 [34-69]	0.04*
CD8$^+$ as % of T cells	55 [27-65]	25 [19-33]	26 [20-34]	29 [21-53]	34 [25-66]	0.04*
PD1$^+$ as % of T cells	6 [5-22]	22 [14-35]	13 [6-25]	12 [6-19]	5 [1-29]	0.001*
• % CD4$^+$	88 [69-96]	85 [77-90]	89 [81-94]	83 [82-90]	Few events	0.34
• % CD8$^+$	12 [5-32]	15 [10-22]	11 [7-19]	17 [10-19]	Few events	0.40
• % CD45Ra$^+$	26 [12-51]	8 [3-17]	12 [4-31]	8 [4-17]	Few events	0.08
• % CD45Ro$^+$	68 [45-88]	92 [82-97]	88 [69-96]	92 [83-96]	Few events	0.06
• % CD103$^+$	6 [2-8]	8 [4-18]	5 [2-14]	13 [3-21]	Few events	0.27
FoxP3$^+$CD25$^+$ as % of CD4$^+$ T cells	8 [4-9]	15 [12-21]	16 [13-24]	17 [9-24]	19 [12-20]	0.001*

Values expressed as median percentages [Interquartile range]; CD, Crohn's disease; HC, Healthy control; ImC, Immunotherapy-related colitis; InfC, infectious colitis; UC, ulcerative colitis. * Significant p-value ≤0.05, determined by using Kruskal-Wallis testing.

Figure 2 *Representative flowcytometric analyses of PD-1$^+$ within CD3$^+$ T cells on colonic biopsies of HC (A), UC patient (B), CD patient (C), InfC patient (D) and ImC patient (E) taken during initial endoscopy. CD, Crohn's disease; HC, Healthy control; ImC, Immunotherapy-related colitis; InfC, infectious colitis; PD-1, Programmed death-1; UC, ulcerative colitis.*

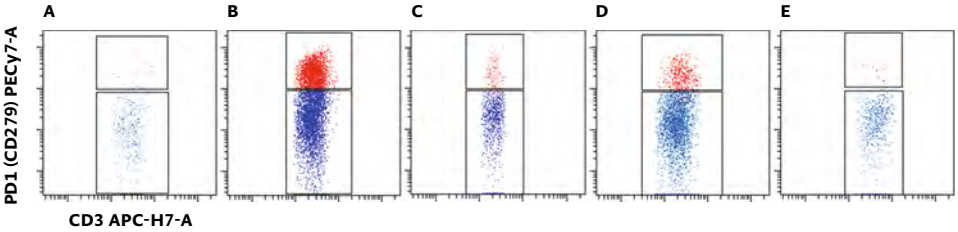
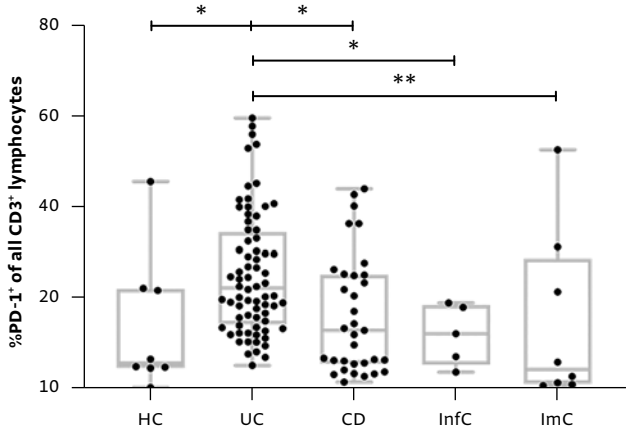


Figure 3 *Percentages of PD-1$^+$ T-cell subsets in colonic biopsies of healthy controls, patients with ulcerative colitis, Crohn's disease, infectious colitis and immunotherapy-related colitis. CD, Crohn's disease; HC, Healthy control; ImC, Immunotherapy-related colitis; InfC, infectious colitis; PD-1, Programmed death-1; UC, ulcerative colitis. * Significant p-value ≤0.05, ** Significant p-value ≤0.01.*

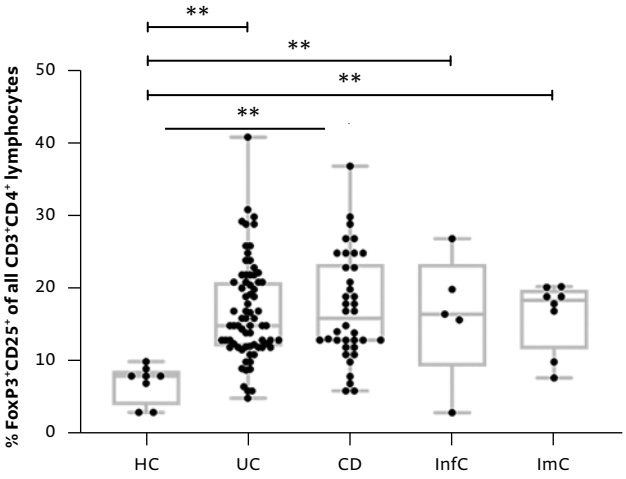


In colonic biopsies of UC patients, statistically significant higher percentages of PD-1$^+$ T cells (22% (14-35)) were found compared to HC (6% (5-22)), CD patients (13% (6-25)), InfC (12% (6-19)) and ImC (5% (1-29), p=0.001). The frequencies of the PD-1$^+$ T-cell subpopulations did not differ among the study groups, except for the memory T-cell subsets. PD-1$^+$CD45Ro$^+$ T cells were more abundant in all different types of colitis compared to HC (respectively 92% (79-97) and 68% (45-88), p=0.06).

Statistically significant higher percentages of Tregs were present in all types of colitis when compared to HC (p=0.001, **Figure 4**). The PD-1/Treg ratio was 1.5 in UC, 0.8 in CD, 0.7 in InfC, 0.3 in ImC and 0.8 in HC. The ratio differs statistically significant between UC and CD (p=0.001) and between UC and ImC (p=0.02).

Patients with ImC showed a different composition of T cells in their colonic biopsies, expressed in higher percentages of $CD8^+$ T cells and lower percentages of $CD4^+$ T cells compared to colitis of other etiologies. When focusing on other T-cell subsets in colonic biopsies of patients with ImC (**Supplementary Table 2**), we demonstrated that 39% (27-67) of all $CD3^+$ T cells was $CD103^+$and 85% (46-96) were $CD45Ro^+$.

Figure 4 *Percentages of $FoxP3^+CD25^+$ of all $CD3^+CD4^+$ T-cell subsets in colonic biopsies of healthy controls, patients with ulcerative colitis, Crohn's disease, infectious colitis and immunotherapy-related colitis. CD, Crohn's disease; HC, Healthy control; ImC, Immunotherapy-related colitis; InfC, infectious colitis; UC, ulcerative colitis. ** Significant p-value ≤0.01.*



Interestingly, a group of three patients with ImC had outlying higher percentages of $PD\text{-}1^+$ T cells compared to other patients with ImC. This group received their last immunotherapy infusion more days prior to ileocolonoscopy compared to the group with lower % $PD\text{-}1^+$ T cells (57 days versus 16 days). Since, the half-life of pembrolizumab and nivolumab is about 15 days, the effect of anti-PD-1 therapy either on blocking the PD-1 binding sites for staining by monoclonal antibodies or by depleting $PD\text{-}1^+$ T cells, will decrease over time.[26,27] When more than twice the half-life of anti-PD-1 antibodies has expired in our cohort, the percentage of $PD\text{-}1^+$ T cells increases to even higher percentages (median 35%) demonstrated in patients with UC (median 22%).

Frequencies of PD-1$^+$ T cells in ileal biopsies in Crohn's disease and healthy controls
To determine whether PD-1$^+$ T-cell subset' distribution was influenced by location of disease, we also investigated these subsets in inflamed ileal biopsies of 36 CD patients (14 of the total 50 CD patients included had normal ileum) and 6 HC in order to compare them with colonic biopsies (**Supplementary Table 3**). There was no difference in the percentage PD-1$^+$ T cells in ileal biopsies of CD patients compared to HC (p=0.63). % PD-1$^+$ T cells in ileal specimens were also comparable to percentages demonstrated in the colonic biopsies of patients with CD (p= 0.86) and HC (p=0.57).

Correlation between PD-1 expression on T cells and disease activity
We investigated the correlation between the number of PD-1$^+$ T cells and markers related to disease activity such as CRP, fecal calprotectin and endoscopic scores (Mayo score for UC and ImC, and SES-CD for CD). The number of PD-1$^+$ T cells was not associated with CRP, calprotectin values and the endoscopic score for UC (respectively p=0.07, p=0.12 and p=0.14), neither for CD (respectively p=0.27, p=0.13 and p=0.86).

Discussion

In the present study, statistical significant differences were demonstrated in percentages of PD-1$^+$ T cells in newly diagnosed UC patients when compared to patients with CD, InfC, ImC and HC. Immunohistochemistry revealed that PD-1 was only expressed in the lamina propria of the colon, not in the epithelial layer. In UC, higher numbers of PD-1 expressing lymphocytes were seen when compared to InfC and ImC, whereas these lymphocytes were absent in HC. Flowcytometric analysis confirmed that UC patients express the highest percentages of PD-1$^+$ T cells compared to moderate percentages seen in CD patients and InfC and low percentages in ImC and HC. In contrast to PD-1, PD-L1 was seen both in the lamina propria and epithelium of the colon, whereas percentages of PD-L1$^+$ lymphocytes in the epithelium were low or completely absent in InfC, ImC and HC, and high in UC. In the lamina propria, PD-L1 was upregulated in UC, InfC and ImC compared to HC.

Different hormones, TCR signaling, cytokines and various transcription factors can stimulate the expression of PD-1 on T cells.[1] PD-1 and its ligands prevent potential autoreactive T cells from causing damage by indirectly stimulating Treg development and directly inhibiting potential pathogenic autoreactive T cells.[1] The PD-1 pathway might reduce the threshold of TGF-b mediated signals needed for the conversion of naive CD4$^+$ T cells into FoxP3$^+$ Tregs which suppress effector T cells and help prevent immune-mediated tissue damage.[1] At first, an upregulation of PD-1 and PD-L1 on infiltrating lymphocytes was seen in mice models with autoimmune diseases.[28] In a murine model, colitis was induced by adoptive transfer of CD4$^+$CD45RBhigh T cells.[15] The inci-

dence of colitis decreased after several repeated tranfers of these T cells into different mice, explained by an accompanied increased expression of PD-1 on these $CD4^+$ T cells in the lamina propria.[15] In this study, PD-1 deficient mice developed more severe CD-like colitis, suggesting that PD-1 mediated inhibitory signals have a regulatory function and limit the severity of colonic inflammation.[15] However, another research group showed that PD-1 deficient mice did not develop colon inflammation explained by alterations in the gut microbiota 16, whereas another group showed that DSS-induced colitis improved through administering A. muciniphila, a gram-negative anaerobic bacterium, which inhibited the activation of cytotoxic lymphocytes through upregulation of PD-1.[17] Results on PD-L1 in animal models are also contradictory, as suppression of PD-L1 in mice reduces chronic intestinal inflammation in one study, 18 whereas others demonstrated that PD-L1-/- mice experienced a worsening of their colitis compared to wild type mice.[19]

A clinical registry study demonstrated that patients with underlying IBD and cancer treated with immunotherapy with PD-1/PD-L1 inhibitors had more gastrointestinal adverse events compared to patients without pre-existing IBD. This suggests that IBD patients are more susceptible to gastrointestinal inflammation following treatment with PD-1/PD-L1 inhibitors, possible because of pre-existent dysfunction of this pathway, despite higher numbers of $PD\text{-}1^+$ lymphocytes in IBD.[29] In IBD patients, Nakazawa et al showed that intestinal epithelial cells expressed PD-L1 both at the mRNA and surface protein level (using flowcytometry), whereas PD-L1 was only observed at mRNA levels in HC.[30] In another study, statistically significant increased PD-1 and PD-L1 expression on $CD3^+$ LP T cells was described in both UC and CD patients using flowcytometry.[18] In the present study we also observed statistically significant PD-1 elevation in UC, but less in CD patients, when compared to HC. Using immunohistochemistry, Cassol et al showed that PD-1 expression was highest in InfC when compared to IBD and HC, while in our immunohistochemistry analysis, PD-1 expression in InfC was lower compared to UC.[31] PD-1 expression in ImC is low in both studies potentially due to the succesfull effect of anti-PD-1 treatment, but we did not find the same increase in PD-L1 expression in the epithelium in ImC as we did for UC patients. A potential explanation for this difference can be due to diversity in the microbiome of included patients and should be investigated in a larger studygroup. Based on this we can not underline potential similarities in the pathogenesis between IBD and ImC as suggested by Cassol et al.[31] Another study in InfC demonstrated an increase in PD-L1 expression which led to decelerated activity of $CD8^+$ T cells and a decrease in Tregs.[32]

Another possible explanation for the difference between our study and other research, is that we investigated inflamed biopsies of a large group of newly diagnosed untreated UC and CD patients, whereas other authors also studied material of patients with inactive disease using immunosuppressive medication.[18] A study of lymphoid patch antigen presenting cells (APC) in CD demonstrated no expression of PD-1 which might also corroborate the difference between UC and CD found in the present research.[33]

We demonstrated that the number of Tregs in colitis of different etiologies was equally elevated compared to percentages found in HC. Although we found significant higher percentages of PD-1$^+$ T cells in UC, this was not accompanied by higher levels of Tregs in UC compared to ImC, InfC and CD. Ongoing inflammation in the presence of a stronger activation of the immunosuppressive system by an elevation of PD-1 is a stronger feature of UC than CD. The upregulation of PD-1 seems to be an inefficient anti-inflammatory counterweight against an obviously stronger immune stimulation in IBD.[34]

In tumor tissue, large infiltration with Tregs can be found. These number of Tregs seem to decrease in tumors following immunotherapy,35-37 but in anti-PD-1-related colitis we found elevated percentages of Tregs in colonic biopsies compared to HC. This can potentially be explained by a selective depletion of a subset of Tregs through blockade of PD-1 and activation of other compensatory immune checkpoint mechanisms like CTLA-4, lymphocyte activation gene-3 (LAG-3), T cell immunoglobulin and mucindomain containing-3 (TIM-3), T cell immunoglobulin and ITIM domain (TIGIT) and V-domain Ig suppressor of T cell activation (VISTA), leading to an increase of Tregs in gut mucosa.[38]

PD-1 blockade leads to proliferation of pre-existing CD8$^+$ T cells, and thus to higher cytotoxic action correlated with tumor regression and potentially to development of adverse events as colitis.[39] We demonstrated higher numbers of CD8$^+$ T cells in ImC compared to InfC, UC and CD. These results are in line with the findings of Coutzac et al, who described a predominance of CD8$^+$ T cells (52.4%) in the lamina propria and epithelium of patients with PD-1 induced colitis.[40]

The percentage CD103$^+$ T cells in ImC 39% (27-67) (**Supplementary Table 2**) was higher compared to percentages found in CD (11% (7-21)) and UC (11% (6-20)).[41] Higher numbers of CD103$^+$ T cells might be seen in association with higher percentage of CD8$^+$ T cells in ImC, while in IBD the T-cell infiltrate is dominated by CD4$^+$ Tcells. Next to this, naive and memory T-cell populations were comparable in colitis of different etiologies.[42]

Affecting the PD-1/PD-L1 pathway could be a promising therapeutic target in IBD since signaling through these receptors could possibly weaken autoimmune responses.[43] In mouse models with DSS-induced and T-cell-induced colitis, administration of PD-L1-Fc leaded to activation of PD-1 activity, thereby regulating CD4$^+$ T cells and modulating DC activation.[44] These PD-1-mediated inhibitory signals led to diminution of colonic inflammation. Administration of Fc proteins in mice models with other autoimmune disease, e.g. reumatoïd arthritis, ameliorated disease activity.[45] In humans, there are analogous therapies approved by the US FDA for the treatment of reumatoïd arthritis, like Abatacept which is a CTLA4-Fc fusion protein stimulating immunosuppressive signals by blocking CD80 and CD86 on antigen presenting cells and prevent costimulatory interaction with CD28 on T cells.[46] There are no published trials investigating the potential application of PD-1 agonists in humans yet, but upcoming phase I trials were announced.[22] Results of the present study offer an argument for this treatment strategy for IBD patients. Since blockade of PD-1 in combination with CTLA-4 seems to

be superior compared to either alone,47 one could expect that combining synergistic agonists will increase the anti-inflammatory effect on T cells and therefore the efficacy in treating IBD. However, a detailed investigation of the function of the PD-1$^+$ T cells in UC and CD is essential, before manipulation of these pathways can be considered as new therapeutic modality in the treatment of IBD.

In conclusion, we demonstrated relevant differences in the distribution and frequencies of mucosal PD-1$^+$ T-cell subsets in patients with newly diagnosed UC, CD, InfC and ImC, supporting the hypothesis that IBD and anti-PD-1 mediated colitis are driven by different immunological pathways. The persistance of active inflammation, despite the higher percentage of PD-1$^+$ T cells in IBD patients, demonstrates that this anti-inflammatory counterweight is insufficient in resolving chronic inflammation, possibly explained by a loss of function.

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Supplementary material

Table S1 *Fluorochromes used for Flowcytrometry all obtained from Becton Dickinson Biosciences USA.*

T-cell marker	CD3	CD4	CD8	CD103	CD45RA	CD45RO	CD25	FoxP3	PD-1
Fluorochrome	APC-H7	PE-Cy7	APC-H7/Cy7	FITC	PE	APC	APC	PE	PE-Cy7

Table S2 *Baseline percentages of different $CD3^+$ T-cell subsets explored with flow cytometry in colonic biopsies of patients with immunotherapy-related colitis.*

Flowcytometry **Colon**	**ImC** **N=8**
$CD4^+$ as % of T cells	61 [34-69]
• % CD45RO	81 [34-93]
• % CD45RA	19 [7-67]
$CD8^+$ as % of T cells	34 [25-66]
• % CD45RO	96 [91-99]
• % CD45RA	4 [1-10]
$CD103^+$ as % of T cells	39 [27-67]
CD45RO as % of T cells	85 [46-96]
CD45RA as % of T cells	14 [4-53]

Values expressed as median percentages [Interquartile range]; ImC, Immunotherapy-related colitis.

Table S3 *Baseline percentages of $CD3^+$ within all lymphocytes, $CD4^+$, $CD8^+$ and PD-1^+ (including different subsets) within $CD3^+$ T lymphocytes and $FoxP3^+CD25^+$ as percentage of $CD3^+CD4^+$ T lymphocytes explored with flow cytometry in inflamed ileal biopsies of patients with Crohn's disease and in healthy controls.*

Flowcytometry **Ileum**	**HC** **n=6**	**CD** **n=36**	**p-value**
$CD4^+$ as % of T cells	37 [21-63]	62 [46-80]	0.05*
$CD8^+$ as % of T cells	63 [37-79]	38 [20-54]	0.05*
$PD1^+$ as % of T cells	8 [4-21]	11[5-17]	0.63
• % $CD8^+$	20 [8.9-65]	17 [7-51]	0.71
• % $CD4^+$	80 [20-91]	83[49-93]	0.71
• % $CD45Ra^+$	40 [12-58]	19 [10-37]	0.28
• % $CD45Ro^+$	60 [45-88]	82 [64-90]	0.22
• % $CD103^+$	17 [2-39]	6 [2-17]	0.45
$FOXP3CD25^+$ as % of $CD4^+$ T cells	3 [3-6]	17 [7-19]	0.02*

Values expressed as median percentages [Interquartile range]; CD, Crohn's disease; HC, Healthy control.
* Significant p-value ≤0.05, determined by using Mann-Whitney-U testing

CHAPTER 8

General discussion

The aim of this thesis was to investigate the presence of different (potential) immunological treatment targets, including MAdCAM-1 and PNAd expression on endothelial cells next to α4β7, αEβ7 and PD-1 on T lymphocytes in UC and CD patients and their relation to disease activity (1). Furthermore we investigated the functional profile of $CD8^+$ Trm cells since both proinflammatory and regulatory functions have been suggested in former publications(2). Ultimately, we studied the response predictive value of MAdCAM-1 and PNAd expression on endothelial cells and α4β7/αEβ7 on T lymphocytes in UC (3). Introduction of such predictive mucosal biomarkers into clinical practice will enable a more patient tailored care.

In this chapter, I will address the outcomes and focus on the implications of the included studies. In addition, I will propose research questions for future studies. To guide this discussion, **Table 1** provides an overview of the main findings, implications, and limitations of the individual studies.

Findings of this thesis

In chapter 2, we demonstrated that new formation of mucosal $PNAd^+$ HEVs is associated with the presence and histologic severity of inflammation in a large cohort UC patients. $PNAd^+$ venules in the inflamed colonic mucosa at diagnosis disappeared in remission at follow-up. In healthy controls, these $PNAd^+$ venules were completely absent comparable to patients with no active inflammation. Higher proportions of $PNAd^+$ HEVs were associated with more colonic follicles, suggesting formation of tertiary lymphoid organs in the inflamed gut mucosa. The proportion of $MAdCAM\text{-}1^+$ venules in colonic biopsies was higher in UC patients with active inflammation compared to healthy controls. A higher proportion of these $MAdCAM\text{-}1^+$ venules seem to be predictive for the number of exacerbations and increases with each subsequent exacerbation.

In the prospective study of chapter 3, we showed that the percentages of $\alpha4\beta7^+CD3^+$ lymphocytes and $MAdCAM\text{-}1^+$ venules in colonic biopsies before initiating treatment with Vedolizumab are significantly higher in biological naïve UC patients responding to Vedolizumab at week 16 compared to non-responders. These findings strengthen the approach of assessing integrin-related-mucosal biomarkers such as $\alpha4\beta7^+CD3^+$ lymphocytes and $MAdCAM\text{-}1^+$ venules before initiating treatment with Vedolizumab.

In chapter 4, substantial differences in the intestinal αE^+ T lymphocyte subsets of patients with active CD and UC compared with healthy controls and patients in endoscopic remission were seen. In active inflammation the percentages of αE- T-cell subsets were higher compared with healthy controls and IBD patients with no inflammation, with the majority consisting of $\alpha E\text{-}CD4^+$ T cells. Both αE^+CD4^+ and αE^+CD8^+ T cells were decreased in active IBD, with a more pronounced decrease within the $CD8^+$ subset. In patients with endoscopic remission during follow-up, the proportion of αE^+CD4^+ and αE^+CD8^+ T cells increased to levels comparable to HCs.

T cells homed to the lamina propria and epithelium can become tissue-resident memory T (Trm) cells upon expression of the Trm cell markers CD69 and αE. Since available data are conflicting regarding the profile of human $CD8^+$ Trm cells, with studies suggesting both proinflammatory and regulatory functions, we investigated the functional profile of these cells in the context of therapeutic strategies targeting gut Trm cells in Chapter 5. We demonstrated that intestinal $CD8^+$ Trm cell transcription profiles depend on their mucosal localization. Lamina propria located αE^+CD8^+ T cells have a classical Trm cell profile with active pathways for regulating longevity and cytokine signaling, while intraepithelial αE^+CD8^+ T cells actively sense the external environment as part of the mucosal barrier and display enrichment in natural killer receptors and innate-like markers. The changes seen during active inflammation are more pronounced in the intraepithelial αE^+CD8^+ T cell subset, leading to an innate proinflammatory profile with a concurrent loss of homeostatic functions.

In chapter 6a we react on assumptions made by Ichikawa and colleagues.[1] They suggest that αE levels can be used as a biomarker in selecting patients suitable for Etrolizumab (anti-integrin β7) treatment based on their potentially pro-inflammatory role, higher levels in ileum compared to colon, and stability of these cells regardless of inflammation and medication use. However the largest pro-inflammatory mucosal T cell subset in IBD consists of αE-T cells. The subset, mentioned by Ichikawa and colleagues, αE^+CD4^+ represents a minority of αE^+ T cells. The majority is αE^+CD8^+ and these cells have an immunosurveillance and protective function in the lamina propria. They described no difference in αE levels between HC and IBD patients, nor any correlation with clinical, endoscopic or histological disease activity, questioning the use of αE levels as a biomarker in IBD.

In chapter 6b, we mentioned it would be interesting to analyze αE^+ T cells in the epithelium and lamina propria separately in order to study the possible role of αE^+ T cells in the pathophysiology of IBD.

In chapter 7 we demonstrated relevant differences in percentages PD-1^+ T cells in newly diagnosed UC patients when compared to patients with CD, infectious colitis, immunotherapy-related colitis and healthy controls. Flowcytometric analysis revealed that UC patients express the highest percentages of PD-1^+ T cells compared to moderate percentages seen in CD patients and infectious colitis, and low percentages in immunotherapy-related colitis and healthy controls.

Strengths and limitations

The strength of the study in chapter 2 is that it comprises a large cohort of newly diagnosed UC patients with serial measurements during remission and active disease phases, revealing the evolution in time of the vascular addressins. The value of $PNAd^+$ and MAdCAM-1^+ venules as treatment targets, biomarkers in predicting response to therapy

and the effect of different treatments on the development and function of these venules has not been studied in chapter 2.

Chapter 3' strength is that we prospectively investigated UC patients with moderate to severe disease activity without concomitant use of corticosteroids or previous exposure to anti-TNFα therapy. Besides, we studied mucosal instead of serological biomarkers, providing a more accurate reflection of the target molecule on the site of inflammation. The relative small sample size of the present cohort might be considered as a limitation, but still we found significant results that support our hypothesis. Methods as immunophenotyping by flowcytometry are challenging to implement in daily practice since biopsies must be investigated within a few hours after withdrawal and are quite expensive techniques. Since immunohistochemistry is more widely used in endoscopic centers and less costly, its clinical utility is superior to flowcytometry. Therefore for example the measurement of MAdCAM-1 on endothelial venules by immunohistochemistry might improve the feasibility of response predictors for Vedolizumab in the daily practice.

A limitation of chapter 4 might be the use of intestinal biopsies that do not reach the deepest layers of the intestinal wall such as the muscularis layer. This might lead to an underestimation of the T-cell infiltration of the deepest layers in CD patients with transmural inflammation. The mechanical method used to pre-process the biopsy specimens before flowcytometry might have had an influence on the absolute cell numbers. However in a comparison of different methods (mechanical, enzymatic and organ culture protocols), they all proved to have their limitations. In line with this method, we were not able to distinguish the lymphocyte infiltrate in lamina propria from epithelium. The strength of this prospective study is that at the time of inclusion, all IBD patients were naïve to immunosuppressive treatment (e.g. steroids, thiopurines, aminosalicylates and biologics).

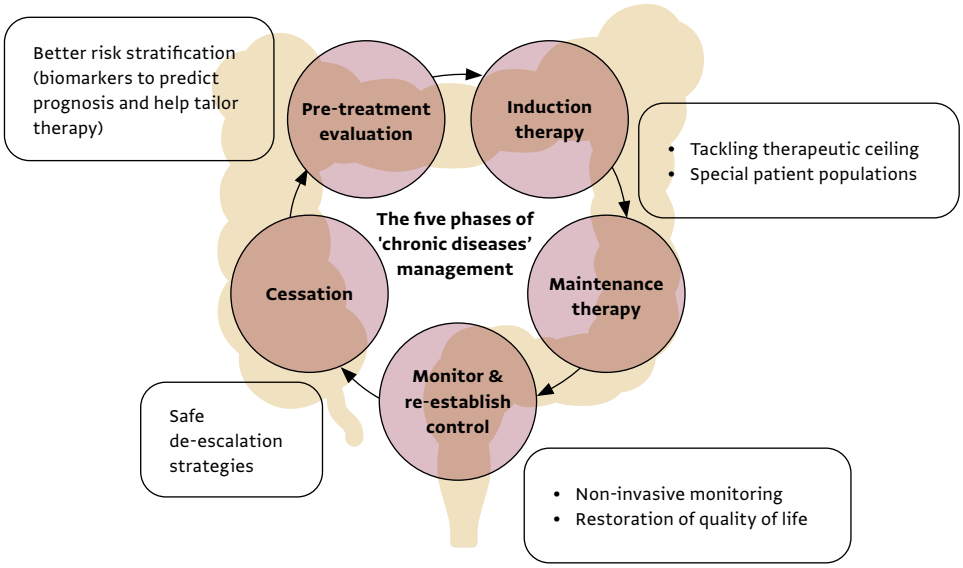
In chapter 5, we performed for the first time imaging mass cytometry, flow cytometry and RNA-sequencing to compare functional profiles of lamina propria and intraepithelial αE^+/-$CD69^+CD8^+$ T cells in healthy control subjects and patients with active ileal Crohn's disease. Unfortunately we could not study the αE^+/-$CD69^+CD4^+$ T-cell subset, which have been correlated with disease flares in IBD in the past.

A limitation of chapter 7 is the lack of a detailed investigation of the function of the PD-1^+ T cells in UC and CD. This is necessary before manipulation of these pathways can be considered as new therapeutic modality in the treatment of IBD.

Implications and future perspectives

Despite great advances in the modern management of IBD with the introductions of new effective drugs, adoption of stricter endpoints and use of better treatment strategies, there remain many unmet needs (See **Figure 1**):

Figure 1 *The five phases of chronic diseases' management and unmet needs in IBD.*[3]



1 Better risk stratification, biomarkers to predict prognosis and help tailor therapy.
2 The challenge remains to be able to predict those IBD patient who will benefit most from early intensive therapy to prevent progression of disease and avoid disease complications, while sparing those who will derive minimal benefit from such approach.[4]
3 Choosing the best therapy for the individual patient. IBD is a heterogeneous multi phenotypic disease, despite this, current treatment algorithms suggest a standard approach to all patients without considering individual risk factors and molecular specificities of disease. The 'one size fits all' approach in treating IBD patients' needs to be abandoned.[5]
4 Therapeutic ceiling and treatment sequencing. Despite all treatment options remission rates are still less than 50%, revealing a therapeutic ceiling in the management of IBD.[6] Since many treatment options enter the market, questions regarding the sequence in which drugs should be used persist.
5 Better monitoring. Identifying failure of therapy sooner leads to an earlier adaptation of treatment to achieve long term remission. Disease monitoring must increasingly focused on non-invasive strategies to avoid costs and inconvenience for patients.
6 When and how to de-escalate therapy once remission is achieved.

The findings in chapter 2 imply that PNAd expression is of diagnostic value in UC, since it appears when gut mucosa is inflamed and disappears completely in remission. In clinical practice, diagnosis of UC is established by fulfilling clinical and endoscopical criteria. Presence of PNAd can be an affirmative histological criterium in the future. Especially when some patients have clinical complaints, but do not have obvious endoscopic disease activity, checking expression of PNAd in biopsy specimens could confirm whether disease is histological active or not. Future research might elucidate the use of PNAd expression in differentiating between different IBD and other types of colitis such as ischemic colitis, diverticulitis, infectious colitis. We also demonstrated a significant correlation between the proportion $PNAd^+$ venules and the number of lymphoid follicles. In healthy controls, $PNAd^+$ venules are only seen in secondary lymphoid tissue in the gut and absent in extrafolliculair mucosal tissue. The presence of these extrafolliculair $PNAd^+$ HEVs in active UC suggests de novo formation of tertiary lymphoid organs thereby controlling the influx and potential local activation of T cells. In future research it would be relevant to investigate the involvement of PNAd and different chemokines in the formation of TLOs and the potential use of these markers as therapeutic targets in IBD. Chapter 2 revealed that a higher proportion of $MAdCAM\text{-}1^+$ venules at baseline was associated with more exacerbations during clinical follow-up. These $MAdCAM\text{-}1^+$ venules can be used as a prognostic biomarker for a worse disease course, identifying patients in need of an aggressive induction treatment strategy. Given the upregulation of MAdCAM-1 over time, based on the number of exacerbations, this supports the hypothesis that treatment with antibodies against MAdCAM-1 (Ontamalimab) could be effective in inducing and maintaining remission in patients with longstanding disease (activity). Chapter 2 contributes to unmet needs 1, 2 and 3.

Chapter 3 demonstrates that pre-treatment percentages of $MAdCAM\text{-}1^+$ venules and $\alpha4\beta7^+$ $CD3^+$ lymphocytes in mucosal biopsies were significantly higher in biological naive UC patients that responded to Vedolizumab compared to non-responders. The patients who priorly failed anti-TNFα therapy with low MAdCAM-1 percentages did not respond to Vedolizumab. Our findings strengthen the approach of assessing mucosal biomarkers such as the frequencies of $\alpha4\beta7^+CD3^+$ lymphocytes and $MAdCAM\text{-}1^+$ venules before initiating treatment with Vedolizumab. Following validation in a larger patient cohort, implementation of these biomarkers related to integrin-dependent homing in clinical practice might lead to a more target based, personalized and cost-effective therapeutic approach in UC. Further work is also needed to investigate whether in Vedolizumab responders T-cell homing to the gut mucosa is more driven by binding of $\alpha4\beta7^+$ $CD3^+$ lymphocytes to MAdCAM-1 and potentially less by interaction of T cells with other adhesion molecules (VCAM-1 and ICAM-1). The use of this alternative pathway in non-responders, may ensure access to the gut in order to maintain or even increase inflammation. Chapter 3 contributes to unmet needs 1, 2, 3 and 4, were Vedolizumab can be given to the patient who benefits the most.

The development of anti-β7 treatment was prompted by the idea of increased $\alpha E^+\beta 7^+$ T lymphocytes in the inflamed intestine of IBD patients. In chapter 4 we found no evidence for an upregulation of αE on intestinal $CD4^+$ and $CD8^+$ T cells during chronic inflammation in time.

Chapter 5 demonstrate the heterogeneity and dual functionality of Trm cell subsets in the intestinal mucosa. Since Etrolizumab affects the adhesion of integrin αE to E-cadherin resulting in decreasing intraepithelial αE^+ cell counts (without distinction for immune cell type)7 and in a reduced accumulation of mainly $CD8^+$ and T helper 9 cells.[8] Thus, primarily the αE^+CD8^+ Trm cell subset seems to be targeted by Etrolizumab. The question is whether this is desirable because $\alpha E^+CD4^+CD69^+$ and not $\alpha E^+CD8^+CD69^+$ T cells have been correlated with disease flares in IBD.[9] Long-term integrin β7 blockade could have a negative impact on the presence, and thus homeostatic functions, of αE^+CD8^+ T cells, which clearly warrants further evaluation. In a large phase 3 trial program in UC, the anti- β7 antibody Etrolizumab yielded disappointing results, supporting our hypothesis.

The chapters 6A and 6B challenge the proposed effect of anti-β7 treatment in IBD patients since most T lymphocytes in inflamed mucosa were $\alpha E\text{-}CD4^+$. The numbers of αE^+ T lymphocytes in active inflamed mucosa were low and the majority consisted of $CD8^+$ T cells with a suggested immunosurveillance and protective function. We think that blocking these αE^+CD8^+T cells with anti-β7 treatment would do more harm than good. But even this is debatable, since there is no evidence that blocking the binding of αE cells to E-cadherin will target the function of the intestinal T lymphocytes. In order to study the possible role of αE^+ T cells in the pathophysiology of IBD, it would be interesting to analyze these cells in the epithelium and lamina propria separately.

Chapter 7 supports the hypothesis that IBD and anti-PD-1 mediated colitis are driven by different immunological pathways. The persistence of active inflammation, despite the higher percentage of $PD\text{-}1^+$ T cells in IBD patients, demonstrates that this anti-inflammatory counterweight is insufficient in resolving chronic inflammation, possibly explained by a loss of function. Affecting the PD-1/PD-L1 pathway could be a promising therapeutic target in IBD since signaling through these receptors could possibly weaken autoimmune responses. There are no published trials investigating the potential application of PD-1 agonists in humans yet, but upcoming phase I trials were announced. Since blockade of PD-1 in combination with CTLA-4 seems to be superior compared to either alone one could expect that combining synergistic agonists will increase the anti-inflammatory effect on T cells and therefore the efficacy in treating IBD. However, a detailed investigation of the function of the PD-1 T cells in UC and CD is essential before manipulation of these pathways can be considered as new therapeutic modality in the treatment of IBD.

Conclusion

The therapeutic arsenal for IBD is rapidly expanding, with multiple novel agents awaiting to enter the clinical practice. This makes it challenging to choose the most appropriate drug for the individual patient. The treatment strategy of IBD patients is shifting from a symptom based approach to a more treat-to-target method. In this thesis we investigated several pre-treatment mucosal biomarkers in IBD patients and studied their behavior/expression in relation to the severity of inflammation, location of disease and disease course over time. These biomarkers might be of help in implementing treat-to-target strategies in IBD. In the future, prospective validation studies including homogeneous groups of different IBD phenotypes need to be performed in order to realize implementation of these pre-treatment mucosal biomarkers in daily practice.

Table 1 *Summary of the individual studies in this thesis*

Chapter	Aim(s)	Main findings and conclusion	Limitations and comments
Part I Homing of T cells in ulcerative colitis			
2	To investigate the presence of $PNAd^+$ high endothelial venules (HEVs) and $MAdCAM\text{-}1^+$ venules in the intestinal mucosa of newly diagnosed patients with ulcerative colitis	• Formation of mucosal $PNAd^+$ HEVs is associated with the presence and histologic severity of inflammation in UC • Higher proportion $PNAd^+$ HEVs were associated with more colonic follicles, suggesting formation of tertiary lymphoid organs in active inflammation • Higher proportion $MAdCAM\text{-}1^+$ venules were seen in active inflammation and can potentially predict the number of exacerbations in the future	The effect of different treatments on the development and function of these venules can not be studied because of the retrospective design of the study.
3	Investigate the response predictive value of different pre-treatment mucosal markers related to the integrin-dependent T-lymphocyte homing	Percentages of $\alpha4\beta7^+CD3^+$ lymphocytes and $MAdCAM\text{-}1^+$ venules in colonic biopsies before initiating treatment with Vedolizumab are higher in biological naïve UC patients responding to Vedolizumab compared to non-responders	• The relative small sample size of the present cohort • Immunophenotyping by flowcytometry is challenging to implement in daily practice
Part II Resident T-cell population in IBD			
4	Study percentages of $\alpha E\beta7^+$ T lymphocytes in intestinal biopsies of newly diagnosed untreated IBD patients at baseline and during follow-up and compare them to healthy controls	• In active inflammation the percentages of αE- T-cell subsets were higher compared with HCs and IBD patients with no inflammation, with the majority consisting of $\alpha E\text{-}CD4^+$ T cells • In patients with endoscopic remission during follow-up, the proportion of αE^+CD4^+ and αE^+CD8^+ T cells increased to levels comparable to HCs	The use of intestinal biopsies that do not reach the deepest layers of the intestinal wall

5	To investigate the functional profile of human tissue-resident memory T (Trm) lymphocytes, both of the CD4 and CD8 lineage	Intestinal $CD8^+$ Trm cell transcription profiles depend on their mucosal localization: • Lamina propria located αE^+CD8^+ T cells have a classical Trm cell profile • Intraepithelial αE^+CD8^+ T cells actively sense the external environment as part of the mucosal barrier and display enrichment in natural killer receptors and innate-like markers	Lack of the functional profile of αE^+/-$CD69^+CD4^+$ T-cell subset
6a and 6b	Questioning the potential pathological role of $\alpha E\beta7^+$ T lymphocytes in IBD patients and their value as a biomarker for the use of anti-β7 treatment	-	-
7	The presence and role of $PD1^+$ mucosal T lymphocytes in colitis of different etiologies: IBD, infectious colitis and immunotherapy-related colitis	IBD and anti-PD-1 mediated colitis are driven by different immunological pathways	The lack of a detailed investigation of the function of the $PD\text{-}1^+$ T cells in UC and CD

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APPENDICES

Het doel van dit proefschrift was om de aanwezigheid van verschillende (potentiële) immunologische behandeldoelen te onderzoeken, waaronder de expressie van MAdCAM-1 en PNAd op endotheelcellen naast α4β7, αEβ7 en PD-1 op T lymfocyten in patiënten met colitis ulcerosa (CU) en de ziekte van Crohn (CD) en hun relatie met ziekteactiviteit (1). Verder hebben we het functionele profiel van $CD8^+$ Trm-cellen onderzocht, aangezien zowel pro-inflammatoire als regulerende functies in eerdere publicaties zijn gesuggereerd(2). Uiteindelijk hebben we de voorspellende waarde van MAdCAM-1 en PNAd expressie op endotheelcellen en α4β7/αEβ7 op T-lymfocyten in UC bestudeerd (3). De introductie van dergelijke voorspellende mucosale biomarkers in de klinische praktijk zal zorg op maat van de patiënt mogelijk maken.

In dit hoofdstuk ga ik in op de uitkomsten en focus ik op de implicaties van de opgenomen onderzoeken. Daarnaast stel ik onderzoeksvragen voor toekomstige studies voor.

Bevindingen van dit proefschrift

In hoofdstuk 2 hebben we aangetoond dat nieuwvorming van mucosale $PNAd^+$ HEVs geassocieerd is met de aanwezigheid en histologische ernst van inflammatie in een groot cohort patiënten met colitis ulcerosa. $PNAd^+$ venulen in de ontstoken mucosa van het colon bij diagnose verdwenen in remissie bij follow-up. Bij gezonde controles waren deze PNAd+ venulen volledig afwezig, vergelijkbaar met patiënten zonder actieve inflammatie. Hogere percentages $PNAd^+$ HEVs waren geassocieerd met meer follikels in het colon, wat wijst op de vorming van tertiaire lymfoïde organen in de ontstoken mucosa. Het aandeel $MAdCAM\text{-}1^+$ venulen in colonbiopten was hoger bij CU patiënten met actieve ontsteking in vergelijking met gezonde controles. Een groter deel van deze $MAdCAM\text{-}1^+$ venulen lijkt voorspellend te zijn voor het aantal exacerbaties en neemt toe bij elke volgende exacerbatie.

In de prospectieve studie van hoofdstuk 3 hebben we aangetoond dat de percentages van $\alpha4\beta7^+CD3^+$ lymfocyten en $MAdCAM\text{-}1^+$ venulen in colonbiopten vóór aanvang van de behandeling met Vedolizumab significant hoger zijn bij biologisch naïeve CU patiënten die in week 16 op Vedolizumab respondeerden in vergelijking met non-responders. Deze bevindingen versterken de benadering van het beoordelen van integrinegerelateerde mucosale biomarkers zoals $\alpha4\beta7^+CD3^+$ lymfocyten en $MAdCAM\text{-}1^+$ venulen voordat behandeling met Vedolizumab wordt gestart.

In hoofdstuk 4 werden substantiële verschillen gezien in de intestinale αE^+ T-lymfocyt subsets van patiënten met actieve CD en CU in vergelijking met gezonde controles en patiënten in endoscopische remissie. Bij actieve inflammatie waren de

percentages αE⁻ T-cel subsets hoger in vergelijking met gezonde controles en IBD patienten zonder inflammatie, waarbij de meerderheid bestond uit αE⁻CD4⁺ T cellen. Zowel αE⁺CD4⁺ als αE⁺CD8⁺ T cellen waren verlaagd in actieve IBD, met een meer uitgesproken afname binnen de CD8⁺ subset. Bij patiënten met endoscopische remissie tijdens de follow-up nam het aandeel αE⁺CD4⁺ en αE⁺CD8⁺ T cellen toe tot niveaus die vergelijkbaar zijn met gezonde controles.

T cellen die zich in de lamina propria en het epitheel bevinden, kunnen tissue-resident memory T (Trm) cellen worden na tot expressie komen van de Trm-celmarkers CD69 en αE. Aangezien beschikbare literatuur tegenstrijdig is met betrekking tot het profiel van menselijke CD8⁺ Trm cellen, met studies die zowel pro-inflammatoire als regulatoire functies suggereren, hebben we het functionele profiel van deze cellen onderzocht in de context van therapeutische strategieën gericht op mucosale Trm cellen in Hoofdstuk 5. We hebben aangetoond dat intestinale CD8⁺ Trm cel transcriptieprofielen afhankelijk zijn van hun mucosale lokalisatie. Lamina propria gelokaliseerde αE⁺CD8⁺ T cellen hebben een klassiek Trm profiel met actieve pathways voor het reguleren van de levensduur en cytokinesignalering, terwijl intra-epitheliale αE⁺CD8⁺ T cellen actief de externe omgeving waarnemen als onderdeel van de mucosale barrière en verrijking vertonen in natural killer-receptoren en innate-like markers. De veranderingen die worden gezien tijdens actieve inflammatie zijn meer uitgesproken in de intra-epitheliale αE⁺CD8⁺ T-cel subset, wat leidt tot een aangeboren pro-inflammatoir profiel met een gelijktijdig verlies van homeostatische functies.

In hoofdstuk 6a reageren we op aannames van Ichikawa en collega's.[1] Zij suggereren dat αE levels kunnen worden gebruikt als biomarker bij het selecteren van patienten die geschikt zijn voor behandeling met Etrolizumab (anti-integrine β7) op basis van hun potentieel pro-inflammatoire rol, hogere niveaus in het ileum vergeleken met het colon, en stabiliteit van deze cellen ongeacht inflammatie en medicatie gebruik. De grootste pro-inflammatoire mucosale T-cel subset bij IBD bestaat echter uit αE⁻ T-cellen. De subset, genoemd door Ichikawa en collega's, αE⁺CD4⁺ vertegenwoordigt een minderheid van αE⁺ T-cellen. De meerderheid is αE⁺CD8⁺ en deze cellen hebben een immunosurveillance en beschermende functie in de lamina propria. Ze beschreven geen verschil in αE levels tussen gezonde controles en IBD patiënten, noch enige correlatie met klinische, endoscopische of histologische ziekteactiviteit. Dit brengt het gebruik van αE levels als biomarker in IBD in twijfel.

In hoofdstuk 6b vermeldden we dat het interessant zou zijn om αE⁺ T cellen in het epitheel en lamina propria afzonderlijk te analyseren om de mogelijke rol van αE⁺ T cellen in de pathofysiologie van IBD te bestuderen.

In hoofdstuk 7 hebben we relevante verschillen aangetoond in percentages PD-1⁺ T cellen tussen nieuw gediagnosticeerde colitis ulcerosa patiënten en patiënten met de ziekte van Crohn, infectieuze colitis, immunotherapie-gerelateerde colitis en gezonde controles. Flowcytometrische analyse onthulde dat CU patiënten de hoogste percen-

tages PD-1$^+$ T-cellen tot expressie brengen in vergelijking met gemiddelde percentages die worden gezien bij CD patiënten en infectieuze colitis, en lage percentages bij immunotherapie-gerelateerde colitis en gezonde controles.

Sterke punten en beperkingen

De kracht van de studie in hoofdstuk 2 is dat het een groot cohort van nieuw gediagnosticeerde CU patiënten omvat met seriële metingen tijdens remissie en actieve ziekte, die de ontwikkeling van de vasculaire addressines onthullen over de tijd. De waarde van PNAd$^+$ en MAdCAM-1$^+$ venulen als behandeltargets, biomarkers bij het voorspellen van respons op therapie en het effect van verschillende behandelingen op de ontwikkeling en functie van deze venulen is niet onderzocht in hoofdstuk 2.

De kracht van hoofdstuk 3 is dat we prospectief CU patiënten met matige tot ernstige ziekteactiviteit hebben onderzocht zonder gelijktijdig gebruik van corticosteroïden of eerdere blootstelling aan anti-TNFα therapie. Bovendien bestudeerden we mucosale in plaats van serologische biomarkers, wat een meer accurate weerspiegeling van het targetmolecuul op de plaats van inflammatie opleverde. De relatief kleine sample size van het huidige cohort kan als een beperking worden beschouwd, maar toch vonden we significante resultaten die onze hypothese ondersteunen. Methoden als immunofenotypering door flowcytometrie zijn uitdagend om in de dagelijkse praktijk te implementeren, omdat biopten binnen een paar uur na afname moeten worden onderzocht en het een vrij dure techniek betreft. Aangezien immunohistochemie op grotere schaal wordt gebruikt in endoscopische centra en minder kostbaar is, is het klinische nut ervan superieur aan flowcytometrie. Daarom zou bijvoorbeeld het aantonen van MAdCAM-1 op endotheliale venulen met behulp van immunohistochemie, de haalbaarheid van het implementeren van responspredictoren voor Vedolizumab in de dagelijkse praktijk kunnen verbeteren.

Een beperking van hoofdstuk 4 zou het gebruik kunnen zijn van darmbiopten die niet de diepste lagen van de darmwand bereiken, zoals de muscularis laag. Dit zou kunnen leiden tot een onderschatting van de T-celinfiltratie van de diepste lagen bij CD patiënten met transmurale ontsteking. De mechanische methode die werd gebruikt ter voorbereiding van biopten vóórafgaand aan flowcytometrie, kan van invloed zijn geweest op het absolute aantal cellen. Bij een vergelijking van verschillende methoden (mechanische, enzymatische en organ culture protocollen) bleken ze allemaal hun beperkingen te hebben. Vanwege het gebruik van de mechanische methode konden we het lymfocyteninfiltraat in de lamina propria niet onderscheiden van het epitheel. De kracht van deze prospectieve studie is dat op het moment van inclusie alle IBD patiënten nog niet eerder immunosuppressieve behandelingen hadden ondergaan (bijv. steroïden, thiopurines, aminosalicylaten en biologicals).

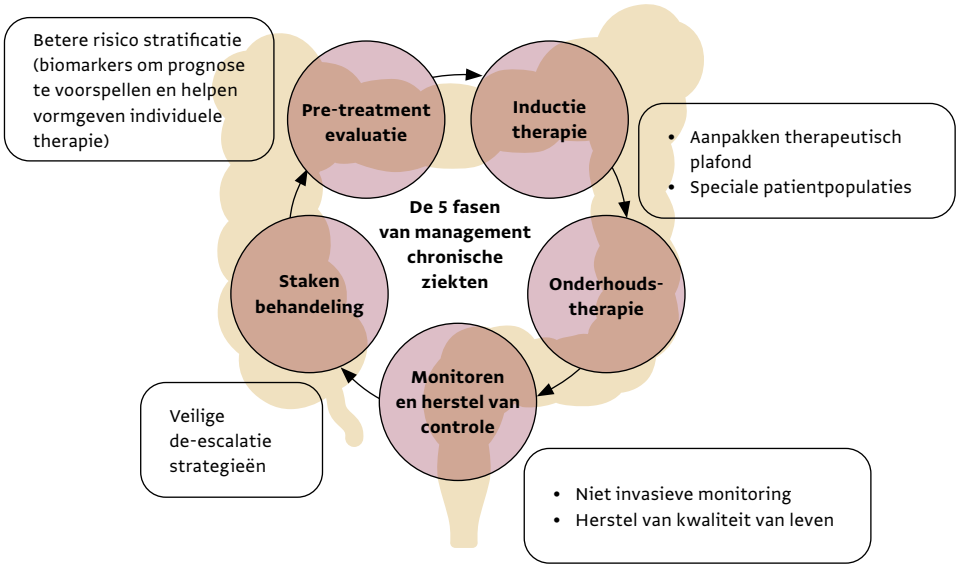
In hoofdstuk 5 hebben we voor het eerst mass cytometrie, flowcytometrie en RNA-sequencing uitgevoerd om functionele profielen van lamina propria en intra-epitheliale $\alpha E^{+/-}CD69^{+}CD8^{+}$ T cellen te vergelijken bij gezonde controles en patiënten met Crohnse inflammatie in het ileum. Helaas konden we de $\alpha E^{+/-}CD69^{+}CD4^{+}$ T cel subset, die in het verleden gecorreleerd is met ziekte exacerbaties bij IBD, niet bestuderen.

Een beperking van hoofdstuk 7 is het ontbreken van een gedetailleerd onderzoek naar de functie van de PD-1^{+} T cellen in CU en CD. Dit is nodig voordat manipulatie van deze pathways kan worden beschouwd als een nieuwe therapeutische modaliteit bij de behandeling van IBD.

Implicaties en toekomstperspectieven

Ondanks grote vooruitgang in de moderne behandeling van IBD met de introductie van nieuwe effectieve medicijnen, de toepassing van striktere eindpunten en het gebruik van betere behandelstrategieën, blijven er veel onvervulde behoeften bestaan (zie **Figuur 1**)[2]:

Figuur 1 *De vijf fasen van het managen van chronische ziekten en onvervulde behoeften in IBD.*[3]



1 *Betere risicostratificatie, biomarkers om prognose te voorspellen en therapie op maat*. De uitdaging blijft om te kunnen voorspellen welke IBD patiënt het meeste baat zal hebben bij vroege intensieve therapie om progressie van de ziekte en ziektecomplicaties te voorkomen, terwijl degenen worden gespaard die minimaal baat zullen hebben bij een dergelijk intensieve aanpak.[4]
2 *De beste therapie kiezen voor de individuele patiënt*. IBD is een heterogene multifenotypische ziekte, desondanks suggereren de huidige behandelingsalgoritmen een standaardbenadering voor alle patiënten zonder rekening te houden met individuele risicofactoren en moleculaire specificiteiten van de ziekte. De 'one size fits all'-benadering bij de behandeling van IBD patiënten moet worden losgelaten.[5]
3 *Therapeutisch plafond en behandelvolgorde*. Ondanks alle behandelingsopties is het remissiepercentage nog steeds minder dan 50%, wat wijst op een therapeutisch plafond in de behandeling van IBD.[6] Aangezien er veel behandelingsopties op de markt komen, blijven er vragen bestaan over de volgorde waarin medicijnen moeten worden gebruikt.
4 *Beter toezicht*. Het sneller identificeren van falen van therapie leidt tot een eerdere aanpassing van de behandeling om langdurige remissie te bereiken. Ziektemonitoring moet zich steeds meer richten op niet-invasieve strategieën om kosten en ongemak voor patiënten te voorkomen.
5 *Wanneer en hoe de therapie te de-escaleren zodra remissie is bereikt*.

De bevindingen in hoofdstuk 2 impliceren dat PNAd expressie van diagnostische waarde is in CU, aangezien het tot expressie komt wanneer de mucosa ontstoken is en volledig verdwijnt in remissie. In de klinische praktijk wordt de diagnose van CU vastgesteld door te voldoen aan klinische en endoscopische criteria. De aanwezigheid van PNAd kan in de toekomst een bevestigend histologisch criterium zijn. Vooral wanneer sommige patiënten klinische klachten hebben, maar geen duidelijke endoscopische ziekteactiviteit hebben, zou het controleren van de expressie van PNAd in de biopten kunnen bevestigen of de ziekte histologisch actief is of niet. Toekomstig onderzoek zou het gebruik van PNAd expressie kunnen verhelderen bij het differentiëren tussen IBD en andere soorten colitis zoals ischemische colitis, diverticulitis, infectieuze colitis. We hebben ook een significante correlatie aangetoond tussen het aandeel $PNAd^+$ venulen en het aantal lymfoïde follikels. Bij gezonde controles worden $PNAd^+$ venulen alleen gezien in secundair lymfoïde weefsel in de darm en afwezig in extrafolliculair slijmvliesweefsel. De aanwezigheid van deze extrafolliculaire $PNAd^+$ HEVs in actieve CU suggereert nieuwvorming van tertiaire lymfoïde organen, waardoor de instroom en mogelijke lokale activering van T cellen wordt gecontroleerd. In toekomstig onderzoek zou het

relevant zijn om de betrokkenheid van PNAd en verschillende chemokines bij de vorming van TLOs en het mogelijke gebruik van deze markers als therapeutische targets bij IBD te onderzoeken. Hoofdstuk 2 onthulde dat een hoger percentage MAdCAM-1$^+$ venulen bij baseline geassocieerd was met het frequenter optreden van exacerbaties tijdens de klinische follow-up. Deze MAdCAM-1$^+$ venulen kunnen worden gebruikt als een prognostische biomarker voor een slechter ziekteverloop, waarbij patiënten worden geïdentificeerd die een agressieve inductiebehandelingsstrategie nodig hebben. Gezien de opwaartse regulatie van MAdCAM-1 in de loop van de tijd, gebaseerd op het aantal exacerbaties, ondersteunt dit de hypothese dat behandeling met antilichamen tegen MAdCAM-1 (Ontamalimab) effectief zou kunnen zijn bij het induceren en behouden van remissie bij patiënten met langdurige ziekte. Hoofdstuk 2 draagt bij aan de onvervulde behoeften 1, 2 en 3.

Hoofdstuk 3 laat zien dat de percentages van MAdCAM-1$^+$ venulen en α4β7$^+$CD3$^+$ lymfocyten in mucosale biopsieën voorafgaand aan behandeling significant hoger waren bij biologisch naïeve UC patiënten die respondeerden op Vedolizumab vergeleken met non-responders. De patiënten die eerder faalde op anti-TNFα therapie met lage MAdCAM-1 percentages respondeerden niet op Vedolizumab. Onze bevindingen versterken de benadering van het beoordelen van mucosale biomarkers zoals de frequenties van α4β7$^+$CD3$^+$ lymfocyten en MAdCAM-1$^+$ venulen voordat behandeling met Vedolizumab wordt gestart. Na validatie in een groter patiëntencohort, zou de implementatie van deze biomarkers gerelateerd aan integrine-afhankelijke homing in de klinische praktijk kunnen leiden tot een meer doelgerichte, gepersonaliseerde en kosteneffectieve therapeutische benadering in CU. Verder onderzoek is ook nodig om te onderzoeken of bij responders op Vedolizumab T-cel homing naar de mucosa meer wordt aangestuurd door binding van α4β7$^+$CD3$^+$ lymfocyten aan MAdCAM-1 en mogelijk minder door interactie van T cellen met andere adhesiemoleculen (VCAM-1 en ICAM -1). Het gebruik van deze alternatieve route bij non-responders kan toegang tot de darm verzekeren om zo inflammatie in stand te houden of zelfs te doen verergeren. Hoofdstuk 3 draagt bij aan onvervulde behoeften 1, 2, 3 en 4, waarbij Vedolizumab kan worden gegeven aan de patiënt die er het meeste baat bij heeft.

De ontwikkeling van anti-β7 behandeling werd ingegeven door het idee van verhoogde αE$^+$β7$^+$ T lymfocyten in de ontstoken darm van IBD patiënten. In hoofdstuk 4 vonden we geen bewijs voor een opwaartse regulatie van αE op intestinale CD4$^+$ en CD8$^+$ T cellen tijdens chronische ontsteking in de tijd.

Hoofdstuk 5 demonstreert de heterogeniteit en dubbele functionaliteit van Trm celsubsets in het darmslijmvlies. Aangezien Etrolizumab de adhesie van integrine αE aan E-cadherine beïnvloedt, wat resulteert in een afname van het aantal intra-epitheliale αE$^+$ cellen (zonder onderscheid voor immuunceltype)[7] en in een verminderde accumulatie van voornamelijk CD8$^+$ en T helper 9 cellen.[8] Daardoor lijkt voornamelijk de

αE$^+$CD8$^+$ Trm celsubset het doelwit te zijn van Etrolizumab. De vraag is of dit wenselijk is omdat αE$^+$CD4$^+$CD69$^+$ en niet αE$^+$CD8$^+$CD69$^+$ T cellen gecorreleerd zijn met exacerbaties bij IBD.[9] Langdurige blokkade van integrine β7 zou een negatieve invloed kunnen hebben op de aanwezigheid, en dus homeostatische functies van αE$^+$CD8$^+$ T cellen, wat duidelijk verdere evaluatie rechtvaardigt. In een groot fase 3 onderzoeksprogramma in CU patiënten leverde het anti-β7 antilichaam Etrolizumab teleurstellende resultaten op, wat onze hypothese ondersteunde.

De hoofdstukken 6A en 6B betwisten het voorgestelde effect van anti-β7 behandeling bij IBD patiënten, aangezien de meeste T lymfocyten in ontstoken mucosa αE$^-$CD4$^+$ waren. Het aantal αE$^+$ T lymfocyten in actief ontstoken mucosa was laag en de meerderheid bestond uit CD8$^+$ T cellen met een veronderstelde immunosurveillance en beschermende functie. We denken dat het blokkeren van deze αE$^+$CD8$^+$ T cellen met anti-β7 behandeling meer kwaad dan goed zou doen. Maar zelfs dit is discutabel, aangezien er geen bewijs is dat het blokkeren van de binding van αE cellen aan E-cadherine de functie van de intestinale T-lymfocyten zal aantasten. Om de mogelijke rol van αE$^+$ T cellen in de pathofysiologie van IBD te bestuderen, zou het interessant zijn om deze cellen in het epitheel en de lamina propria afzonderlijk te analyseren.

Hoofdstuk 7 ondersteunt de hypothese dat IBD en anti-PD-1 gemedieerde colitis worden aangestuurd door verschillende immunologische routes. Het aanweizg blijven van actieve ontsteking, ondanks het hogere percentage PD-1$^+$ T cellen bij IBD patiënten, toont aan dat dit ontstekingsremmende tegengewicht onvoldoende is bij het aanpakken van inflammatie, mogelijk verklaard door functieverlies. Het beïnvloeden van de PD-1/PD-L1 route zou een veelbelovend therapeutisch target kunnen zijn in de behandeling van IBD, aangezien signalering via deze receptoren mogelijk de auto-immuunresponsen zou kunnen verzwakken. Er zijn nog geen gepubliceerde onderzoeken naar de mogelijke toepassing van PD-1-agonisten bij mensen, maar er zijn aankomende fase I onderzoeken aangekondigd. Aangezien blokkade van PD-1 in combinatie met CTLA-4 superieur lijkt te zijn in vergelijking met elk afzonderlijk, zou men kunnen verwachten dat het combineren van synergetische agonisten het ontstekingsremmende effect op T cellen en daarmee de werkzaamheid bij de behandeling van IBD zal verhogen. Een gedetailleerd onderzoek van de functie van de PD-1 T cellen in CU en CD is echter essentieel voordat manipulatie van deze routes kan worden beschouwd als een nieuwe therapeutische modaliteit bij de behandeling van IBD.

Conclusie

Het therapeutische arsenaal voor IBD breidt zich snel uit, met meerdere nieuwe middelen die wachten om in de klinische praktijk te worden opgenomen. Dit maakt het een uitdaging om het meest geschikte medicijn voor de individuele patiënt te kiezen.

De behandelstrategie van IBD patiënten verschuift van een op symptomen gebaseerde benadering naar een meer 'treat-to-target' methode. In dit proefschrift hebben we verschillende mucosale biomarkers voor de behandeling bij IBD patiënten onderzocht en hun gedrag/expressie bestudeerd in relatie tot de ernst van de ontsteking, de locatie van de ziekte en het ziekteverloop in de tijd. Deze biomarkers kunnen helpen bij het implementeren van treat-to-target-strategieën bij IBD. In de toekomst moeten prospectieve validatiestudies met homogene groepen van verschillende IBD fenotypes worden uitgevoerd om implementatie van deze pre-treatment mucosale biomarkers in de dagelijkse praktijk te realiseren.

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ABBREVIATIONS

5-ASA	5-aminosalicylic acid
(s)-α4β7	(soluble)-Alfa4beta 7
AUC	Area under the curve
BSA	Bovine serum albumin
CD103	αE, alfa E
CD	Crohn's disease
CRP	C-reactive protein
CTLA-4	Cytotoxic T-lymphoycte-associated protein 4
DSS	Dextran Sulfate Sodium
ERG	ETS related gene
ETS	Erythroblast Transformation Specific
FCS	Fetal calf serum
FDR	False discovery rate
GALT	Gut associated lymphoid tissue
GI	Gastrointestinal
GzmK	Granzyme K
HBSS	Hank's Balanced Salt Solution
HC	Healthy control
HCC	Hepatocellular carcinoma
HEV	High Endothelial Venule
IBD	Inflammatory bowel disease
(s)-ICAM-1	(soluble)-Intercellular adhesion molecule-1
IEC	Intestinal epithelial cells
IEL	Intraepithelial lymphocytes
IFNγ	Interferon gamma
IHC	Immunohistochemistry
IL	Interleukin
IMC	Imaging mass cytometry

ImC	Immunotherapy-related colitis
InfC	Infectious colitis
IQR	Interquartile range
IrAE	Immune-related adverse events
Log2FC	Log2 fold change
LPS	Lipopolysaccharide
(s)-MAdCAM-1	(soluble)-Mucosal vascular Addressin Cell Adhesion Molecule-1
NCI-CTCAE	National Cancer Institute-Common Terminology Criteria for Adverse Events
NES	Normalized enrichment score
NSCLC	Nonsmall cell lung carcinoma
PBMC	Peripheral blood mononuclear cells
PBST	Phosphate-buffered saline containing 0.1% Tween-20
PD-1	Programmed death-1
PD-L1	Programmed death-Ligand 1
PMS	Partial mayo score
PNAd	Peripheral lymph node addressin
ROC	Receiver operating characteristics
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SES-CD	Simple endoscopic score for Crohn's disease
SLO	Secondary Lymphoid Organ
Tcm cells	Central memory T cells
Tem cells	Effector memory T cells
Tn cells	Naïve T cells
Treg cells	Regulatory T cells
TLO	Tertiary Lymphoid Organ
(s)-TNFα	(soluble)-Tumor Necrosis Factor alpha
TRM	Tissue-resident memory T cells
UC	Ulcerative colitis
UCDAI	Ulcerative Colitis Disease Activity Index
(s)-VCAM-1	(soluble)-Vascular cell adhesion molecule-1

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RESEARCH DATA MANAGEMENT

This thesis is based on the results of human studies, which were conducted in accordance with the principles of the Declaration of Helsinki. The medical and ethical review board Committee on Research Involving Human Subjects Region Arnhem Nijmegen, Nijmegen, the Netherlands has given approval to conduct these studies. **Chapter 5** was also approved by the University Medical Center Utrecht.

Data management and monitoring were performed within Research Manager. Paper data were entered into the computer by the use of Research Manager and stored in the department archive of Gastroenterology and Immunology at Rijnstate. The data from patient file research was exported into secondary data sets in Microsoft Excel or IBM SPSS.

After the finalization of each study the data was migrated to a local server in the department of Gastroenterology and Hepatology at Rijnstate Hospital. This server is supported by ICT department of Rijnstate, with daily backups. The privacy of the participants in the studies is warranted by use of encrypted and unique individual subject codes. The subject codes are stored separately from the study data. The data generated or analysed in this thesis are part of published articles and its additional files are available from the corresponding author upon request. The data will be saved for 15 years after the termination of the study.

PHD PORTFOLIO

NAME PHD CANDIDATE	B. Roosenboom
DEPARTMENT	Gastroenterology & Hepatology
GRADUATE SCHOOL	RIMLS
PHD PERIOD	1-1-2017 t/m 1-1-2020
PROMOTOR	Prof. Dr. J.P.H. Drenth
CO-PROMOTORS	Dr. C.S. Horjus Talabur Horje, Dr. E.G. van Lochem, Dr. P.J. Wahab

Training activities	Year(s)	Hours
A **Courses & Workshops**		
• eBROK course	2017	26
• Masterclass biologics - towards personalised medicine Sanquin	2017	10
B **Seminars & lectures**		
• Soeterbeeck GI lectures	2017 - 2020	24
• DDSEP meetings	2017 - 2020	24
• Rijnstate Research Rounds	2017 - 2020	7
• Promovendi Rijnstate Overleg	2017 - 2020	35
• Webinars ICC	2018	8
C **Symposia & congresses**		
• Symposia Immuno-Oncology Rijnstate	2017	5
• ESPEN congress The Hague	2017	8
• EWIMID Utrecht (1 x oral)	2017	24
• ICC-IBD day	2017 - 2020	27
• Dutch Digestive disease days NVGE (2 x oral)	2017 - 2021	91
• ECCO (4 x poster)	2017 - 2021	75
• Young-ICC symposia (1 x oral)	2017 - 2020	30
• ECI congress Amsterdam (1 x poster)	2018	16
• Digestive Disease Week Washington (2 x poster)	2018	10
• Falk IBD congress Lissabon	2018	24
• STZ event Utrecht (1 x oral)	2018	14

D **Teaching activities**		
• Supervision internship (Master student, 3 months)	2017	28
• Supervision internship (ANIOS, 3 months)	2017	28
• Education HAN specialized IBD nurses	2018	14
E **Other**		
• Committee Member and Organization Rijnstate Crohn & Colitis Centre	2017 - 2020	350
• Organisation patient information evening in collaboration with CCUVN (2x)	2018 - 2019	24
• Weekly GI Hep lectures Rijnstate	2017 - 2020	90
• Selection Article of the Year Rijnstate		3
Total		**995**

ABOUT THE AUTHOR

Britt Roosenboom werd geboren op 23 augustus 1992 in Heerlen en groeide op in Doenrade. In 2010 behaalde zij haar Gymnasium diploma aan het Carbooncollege locatie St.-Jan in Hoensbroek. Aansluitend startte ze haar geneeskunde opleiding aan de Radboud Universiteit in Nijmegen. In 2016 vond haar senior co-schap plaats op de Maag-, Darm- en Leverafdeling van het Rijnstate ziekenhuis. Hierna volbracht zij haar wetenschapsstage op de Maag- Darm- en Leverafdeling van het Radboud universitair ziekenhuis, waar zij onder begeleiding van dr. Y. Wouters en dr. G.J.A. Wanten onderzoek deed naar klinische uitkomsten in patienten die totale parenterale voeding krijgen met taurolidine als katheter lock. In januari 2017 begon zij in het Rijnstate ziekenhuis haar promotieonderzoek getiteld 'Mucosal T cells in IBD from immigrants to residents' onder supervisie van copromotoren dr. C.S. Horjus Talabur Horje, dr. E.G. van Lochem en dr. P.J. Wahab (Rijnstate) en promotor prof. dr. J.P.H. Drenth (Radboudumc). Aansluitend werkte zij van januari 2020 als basisarts op de Maag-, Darm-, en Leverafdeling van zowel het Rijnstate ziekenhuis als het Radboudumc. In mei 2021 begon Britt aan de opleiding tot Maag-, Darm- en Leverarts in het Rijnstate te Arnhem bij opleider dr. A.P. Bech (Interne Geneeskunde) en opleiders dr. H.T.J.I. de Leest en dr. M.J.M. Groenen (Maag-, Darm- en Leverziekten). Britt woont samen met Eddy Wichems en hun dochter Ruby in Giesbeek.

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Lieve *Marleen* en *Marcel*, ook jullie wil ik bedanken voor de fijne tijd die jullie mij in het Rijnstate geven. Het is meer dan fantastisch te noemen, dat jullie mij onderdeel lieten voelen van het Rijnstate Crohn en Colitis centrum en kansen gaven om mij te ontwikkelen binnen jullie team.

Paranifmen, mijn lieve zus *Janou* en lieve *Margot*, wat fijn dat jullie naast/achter/voor mij staan wanneer dit ook maar nodig is. Op jullie kan ik werkelijk altijd rekenen! Nu op naar meer vrije tijd samen die niet ook nog deels besteed moet worden aan promotie gerelateerde zaken.

En tot slot, lieve Eddy en Ruby, mama en papa, Janou en Luc, Hermien en Geke, Dorien en Andries, vriendinnen/vrienden, alle arts-assistenten en arts-onderzoekers die gedurende de jaren op mijn pad zijn gekomen, MDL artsen uit het Rijnstate ziekenhuis en het Radboudumc, het hele team van het Rijnstate Crohn en Colitis centrum, iedereen werkzaam op het immunologie lab en de pathologie afdeling van het Rijnstate ziekenhuis, spreekuurassistenten, verpleegkundigen van de MDL afdelingen en polikliniek, endoscopie verpleegkundigen, het MDL secretariaat, opleiders van de interne geneeskunde en de MDL, medewerkers van het leerhuis en niet te vergeten alle IBD patiënten die deel hebben genomen aan de verschillende onderzoeken: dankzij jullie is het gelukt om het onderzoek af te ronden, dankzij jullie bleef ik lachen, dankzij jullie ben ik waar ik nu ben! Veel liefs voor jullie allemaal!

Paranimfen

MARGOT VAN DER HOEK EN JANOU ROOSENBOOM