Immunophenotyping of patients with ulcerative colitis

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Summary

Ulcerative colitis is a chronic inflammatory bowel disease of unknown origin. It involves a complex interaction between adaptive and innate immune system.

The aim of this study was to find prognostic markers in peripheral blood that relate to the inflammatory condition in the patient. To get a better understanding of the dynamics of the inflammatory process, we analysed peripheral blood mononuclear cells of 39 patients with ulcerative colitis (UC) and compared them to those of 24 healthy individuals (Non-UC). Nine of the UC patients had previously undergone colectomy. Except for six patients, all were treated with multiple immunosuppressant drugs. The samples were characterized using 17 cell surface molecules to establish an immunological profile via flow cytometric analysis.

Results identified CD25+ CD4+ cells, CD4+ CRTH2+ cells, CD11b+ cells and CD1a+ CD11b+ cells as biological markers to discriminate between Non-UC and UC donors. The immune profile of colectomised patients was similar to that of other UC patients, indicating that the removal of the main targeted organ does not restore a healthy immune system. This might explain the predisposition of colectomised UC patients to develop pouchitis.

The results from this study corroborate the hypothesis that a comprehensive approach might lead to a better understanding of the immunological processes underlying the pathology of UC. However, future studies will have to be improved regarding subtypes of immune cells and must include the analysis of cytokines and the histologic evaluation of colon tissue. In conclusion, immunological profiling can help us understand the complex mechanisms underlying ulcerative colitis. This can further lead to the identification of more specific targets for drugs and consequently a better and personalized treatment.

Zusammenfassung

Colitis ulcerosa ist eine chronisch entzündliche Darmerkrankung unklarer Genese. Dabei spielt die komplexe Interaktion zwischen angeborenem und erworbenem Immunsystem eine wichtige Rolle.

Das Ziel dieser Studie war es, prognostische Marker im peripheren Blut von Patienten mit Colitis ulcerosa zu finden, die die Entzündungsreaktion widerspiegeln. Um die inflammatorischen Prozesse zu verstehen, haben wir periphere mononukleäre Zellen von 39 Patienten, die an Colitis ulcerosa erkrankt sind, mit denen von 24 gesunden Spendern verglichen. Neun der erkrankten Patienten waren kolektomiert. Mit Ausnahme von sechs Patienten wurde der Rest mit multiplen immunsuppressiven Medikamenten behandelt. Die Proben wurden anhand von 17 Oberflächenmolekülen mittels Durchfluss-Zytometrie charakterisiert, um immunologische Profile zu erstellen.

CD25+ CD4+ Zellen, CD4+ CRTH2+ Zellen, CD11b+ Zellen und CD1a+ CD11b+ Zellen sind als biologische Marker fähig, zwischen Erkrankten und nicht erkrankten Spendern zu unterscheiden. Die Immunprofile von kolektomierten Patienten waren ähnlich zu denen von anderen UC Patienten. Folglich scheint die Entfernung des Zielorgans keine Wiederherstellung eines gesunden Immunsystems zu bewirken. Dies könnte die Prädisposition kolektomierter Patienten zu einer Pouchitis erklären.

Die Ergebnisse dieser Studie bestätigen, dass ein umfassender Ansatz notwendig ist, um die immunologischen Prozesse dieser Erkrankung besser zu verstehen. Insbesondere müssen zukünftige Studien verbessert werden in Betracht auf analysierte Zellen, und sie müssen die Analyse der Zytokine und histologische Untersuchungen des Dickdarms beinhalten. Ein Verständnis der komplexen Mechanismen dieser Erkrankung kann zur Identifizierung von spezifischen Molekülen führen, die wiederum die Möglichkeit zur Entwicklung neuer Therapien bietet und somit die Perspektive für eine bessere und personalisierte Medizin schaffen.

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List of abbreviations

AP	Alkaline phosphatase
CCR2	C-C chemokine receptor type 2
CD	Crohn’s disease
CD	Cluster of differentiation
cDC	Classical dendritic cell
CRP	C-reactive protein
DALM	Dysplasia-associated lesion or mass
DC	Dendritic cell
ESR	Erythrocyte sedimentation rate
FACS	Fluorescence activated cell sorting
FAP	Familial adenomatous polyposis
FoP	Frequency of parent
GGT	Gamma-glutamyltransferase
GWAS	Genome-wide association study
IBD	Inflammatory bowel disease
IFN-γ	Interferon γ
IL	Interleukin
ILC	Innate lymphoid cell
iNKT	Invariant natural killer cell
IPAA	Ileal-pouch anal anastomosis
mm/h	Millimeter per hour
MMX	Multi Matrix System
NK-T cells	Natural killer cells
NOD	Nucleotide-binding oligomerization domain
Non-UC donors	Non-ulcerative colitis donors
p.o.	Per os
p.r.	Per rectum
PAMP	Pathogen associated molecular patterns
PMBC	Peripheral blood mononuclear cell
PRR	Pattern recognition receptor
PSC	Primary sclerosing cholangitis
SCCAI	Simple clinical colitis activity index
SD	Standard deviation
TGF-β	Transforming growth factor β
Th0	Naïve T-cell
Th1 cell	T-helper cell type 1
Th17 cell	T-helper cell type 17
Th2 cell	T-helper cell type 2

TLR	Toll-like receptors
TNF-α	Tumor necrosis factor α
Tregs	Regulatory T-cells
UC	Ulcerative colitis

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1 Literature review

1.1 Definition of ulcerative colitis

Ulcerative colitis is a chronic inflammatory bowel disease that spreads continuously in the superficial layer of the large intestine. [1]

1.2 Epidemiology

In Germany, the incidence of ulcerative colitis is around 6 of 100 000 inhabitants per year, mostly diagnosed between the age of 25 and 35. The risk of developing ulcerative colitis is 15 times higher for siblings compared to the normal population. [1]

1.3 Aetiopathology

The aetiology of UC is still unknown, and it is presently thought that a combination of genetic, environmental and microbial factors contribute to the uncontrolled immune response. Ultimately, UC leads to local tissue damage with erosions, ulcerations and necrosis. Typically, the inflammation starts in the distal rectum and disperses into the proximal colon. [1]

The Montreal classification categorizes the extent of the inflammation (see Table 1) [2]. Early in the inflammatory process, the mucosa is reddish, swollen, bleeds on contact and has small ulcerations. In the chronic stage, the mucosa is damaged and is less folded with loss of the colon haustra. Histologically the early stage shows granulocytes with pus in the crypts, whereas the chronic stage is characterized through mucosal infiltration with lymphocytes and histiocytes. The persistent inflammation can also lead to a mucosal atrophy and dysplasia-associated lesion or mass (DALM), which is considered a precancerous condition. [1]

Table 1: Montreal classification for extent of ulcerative colitis [2]

E1	Ulcerative proctitis	Only rectum
E2	Left sided UC	Distal of splenic flexure
E3	Pancolitis	Extends proximal of splenic flexure

1.4 Symptoms and complications

The cardinal symptom of ulcerative colitis is bloody-slimy diarrhoea accompanied by abdominal pain. This disease is also known for extraintestinal manifestations (see Table 2). As the inflammation continues, it can cause several complications, such as growth disruption in children, weight loss, massive bleeding, toxic megacolon, risk for colorectal cancer and in rare cases amyloidosis. The risk for colorectal cancer correlates with the extent of the colon involvement and the disease duration. If a primary sclerosing cholangitis (PSC) is diagnosed, there is a risk for cholangiocellular carcinoma and the risk for colorectal cancer is even higher. [1]

Table 2: Extraintestinal manifestations of ulcerative colitis [1]

Skin	Erythema nodosum, Pyoderma gangraenosum
Eyes	Iritis, uveitis, episcleritis, conjunctivitis
Musculoskeletal	Arthritis, ankylosing spondylitis
Liver	Primary sclerosing cholangitis (PSC)

1.5 Disease progression

Ulcerative colitis has different forms of disease activity, which is shown in Table 3. [1]

Table 3: Forms of disease activity [1]

Chronic-intermittent disease activity (85%)	Patients experience relapses with phases of complete remission in between
Chronic-continuous disease activity (10%)	Patients experience symptoms of different intensities without phases of full remission
Acute-fulminant disease activity (5%)	Sudden begin of symptoms with cholera-like diarrhoea up to severe dehydration, fever and shock

1.6 Diagnosis

The diagnosis of ulcerative colitis is carried out in multiple steps. A profound anamnesis and physical exam are followed by an inspection of the anus and a digital rectal examination. The diagnosis is confirmed by a complete ileocolonoscopy with multiple biopsies from different locations being necessary. Due to the higher risk of colorectal cancer in UC patients, the ileocolonoscopy also plays an important role in the early detection of cancer. In case of inflammation, an ultrasound could show a thickening of the colon wall. In addition, blood tests indicate an inflammatory condition, such as leucocytosis, C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) elevation. Markers for cholestasis such as gamma-glutamyltransferase (GGT) and alcalic phosphatase (AP) can be increased in case of a PSC. Calprotectin and lactoferrin are two markers, which can be quantified from stool samples to monitor inflammation activity. [1]

1.7 Therapy

1.7.1 Conservative approach

Depending on localisation and intensity of the inflammation, the therapy of ulcerative colitis differs. For evaluation of disease severity, the Truelove and Witts criteria [3] can be used (see Table 4) [4, 5]. The first line of treatment

includes dietary measurements and immune modulating drugs [1, 5]. Table 5 gives an overview of the recommended therapy algorithm.

Table 4: Truelove and Witts criteria for disease severity [3]

Disease activity	Mild	Moderately severe	Severe
Number of stools per day	≤4	Intermediate between severe and mild	≥6
Blood in stool	No more than small amounts of visible blood	Intermediate between severe and mild	Macroscopically visible
Temperature (°C)	Afebrile	Intermediate between severe and mild	>37,5°C (mean evening temperature) or more than >37,8°C on two out of four days
Heart rate	Normal	Intermediate between severe and mild	>90
Anaemia	Not severe	Intermediate between severe and mild	Haemoglobin 75% or less below normal
Erythrocyte sedimentation rate	≤ 30mm/h	Intermediate between severe and mild	> 30mm/h

Table 5: Therapy algorithm in ulcerative colitis [5]

Location of inflammation and severity	Recommended medication	Alternative if refractory
Proctitis - mild to moderate	• Mesalazine p.r.	• Mesalazine p.r. + corticosteroids p.r. • Mesalazine p.r. + Mesalazine p.o.
Left-sided colitis – mild to moderate	• Mesalazine p.r. + Mesalazine p.o.	• Corticosteroids systemically • Budesonide Multi-Matrix-System (MMX)
Extensive disease – mild to moderate	• Mesalazine p.r. + Mesalazine p.o.	• Corticosteroids systemically

Severe UC, independent from disease distribution	• Hospital admission • Corticosteroids systemically	• Intolerance or contraindications to corticosteroids → TNFα-inhibitors, Cyclosporine A or Tacrolimus • Surgical approach should be evaluated
Steroid-refractory UC	• TNFα-inhibitors, Tofacitinib, Cyclosporine A or Tacrolimus • Infliximab should be combined with a thiopurine	• If clinical status deteriorates or shows no improvement under drug therapy, surgery should be considered
Steroid-dependant UC	• Thiopurine, TNFα-inhibitors, Vedolizumab or Tofacitinib	
Maintenance of remission	• Primarily with Mesalazine if UC was responsive to Mesalazine or steroids prior • Mesalazine p.r. or p.o. depending on location of inflammation, or combination • If remission was induced with TNFα-inhibitors, Tofacitinib or Vedolizumab, therapy should be continued with these substances • If remission was induced with calcineurin inhibitors, therapy should be continued with a thiopurine or Vedolizumab • Thiopurines in case of complicated course of disease as first choice	• Higher dose of Mesalazine p.o. and p.r. • TNFα-inhibitors, Vedolizumab or Thiopurine • Methotrexate or calcineurin inhibitors only in exceptional cases

1.7.2 Surgical approach

In cases of acute deterioration, such as toxic megacolon, perforation, bleeding or sepsis, a surgical intervention is inevitable. For patients that suffer from a chronically high disease activity, deterioration of general condition, epithelial dysplasia, growth retardation or local and systemic side effects, a surgery is indicated. So far, the surgical therapy is considered the only option for healing. [1]

The current gold standard is the proctocolectomy with preservation of continence by an ileal pouch-anal anastomosis (IPAA) [6]. Depending on the clinical wellbeing of the patient, the surgery can be performed in two or three procedures. The first step is the proctocolectomy with construction of a terminal ileostomy. The second step covers the creation of the pouch, the ileal pouch-anal anastomosis, under protection by a loop ileostomy. The third step is the reversal of the ileostomy to restore normal bowel passage. The first two steps can be done in one procedure. [7]

Seifarth et al. showed that a three step procedure is recommended in UC patients with distinct immune suppression, as the perioperative morbidity is reduced, the operation times and the hospital stays are shorter [8]. Side effects after IPAA are pouchitis, pouch failure, pouch fistula, pelvic sepsis, incontinence and sexual dysfunction [6]. The most common side effect is pouchitis, which is a non-specific inflammation of the pouch [9]. Nevertheless a long term retrospective analysis from Chile including 116 patients showed that the IPAA preservation rate was 96,5% at 10 years and 93% at 20 years [7]. Overall, most patients regain a quality of life, which is almost as high as in the general population [10, 11].

1.8 Immunological pathways in ulcerative colitis

To date, the origin and pathophysiology of ulcerative colitis is unknown. It is suspected that genetically susceptible individuals combined with environmental factors develop an abnormal immune response, which then

leads to an inflammation [12, 13]. This process is multifaceted and involves the adaptive and innate immune system [13].

The intestinal epithelium is protected by mucus, which consists of an inner firm layer and an outer loose layer [13]. The inner layer is sterile and very dense, whereas the outer layer is more permeable and inhabits bacteria [13]. Underneath the mucus layer lies the intestinal epithelium, consisting of enterocytes and specialized epithelial cells, such as goblet cells and Paneth cells [13]. In inflammatory bowel disease (IBD) impaired epithelial barriers and increased intestinal permeability have been detected [13, 14]. According to genome-wide association studies (GWAS) epithelial barrier impairments are a primary pathogenetic mechanism [13].

1.8.1 Innate immunity in IBD

Epithelial cells and innate immune cells, such as macrophages and dendritic cells (DCs) express pattern recognition receptors (PRRs), which detect pathogen associated molecular patterns (PAMPs) [13]. The term PRR classifies molecules such as trans-membrane Toll-like receptors (TLR) and intracytoplasmic nucleotide-binding oligomerization domain (NOD)-like receptors [13]. These signalling cascades induce nuclear factor (NF)-κB, which leads to production of pro-inflammatory substances [13]. In epithelial cells, this leads to the secretion of antibacterial agents like defensins [13]. Some of these defensins are distributed constantly, whereas others only after PAMP-PRR interaction [13]. Furthermore, PRR activation also triggers antigen presenting cell maturation for adequate T-cell activation and links the innate immune cells to the adaptive immunity [13]. IL-23 also plays an important role in the communication between those two immune cascades [13]. In IBD, single nucleotide polymorphisms in the IL23 receptor gene have been discovered [13, 15]. It can influence Th17 cells and innate cells, such as unconventional T cell populations like γδT cells, invariant natural killer T cells (iNKT), mucosal associated invariant T cells and innate lymphoid cells (ILCs) [13]. Typically, this leads to the production of Th17-related cytokines [13]. In

particular IL-23 activated ILCs were found to be responsible for intestinal inflammation by secretion of IL17A or interferon (IFN)-γ [13, 16].

1.8.2 Adaptive immunity in IBD

In contrast to the innate immune cells, the adaptive immune system is very specialized and enables the development of a lasting immunity. However, an imbalance of both components can lead to an outbreak of an inflammation through an inordinate release of cytokines and chemokines, which have pathogenic effects. Therefore, a fine-tuning of these processes is required with multiple integrated feedback mechanisms. Particularly T-cells play an important role in the disease activity as part of the adaptive immune system. Naïve T cells, Th0, can mature into Th1, Th2 or Th17 cells. [13]

Th1 cells are crucial for eliminating intracellular pathogens and can secrete IFN-γ when induced by IL-12 [13, 17]. IFN-γ, which can be produced by Th1 cells, leads to enterocyte apoptosis and provokes macrophages to secrete TNF-α [13]. This signalling cascade links the adaptive immune system to the innate immune system [13]. The role of TNF-α in IBD is crucial and the inhibition of this cytokine has proven successful in regard of induction and maintenance of mucosal healing in UC and CD compared to placebo [18].

Th2 cells are indispensable for the defence against parasites, arbitrate allergic reactions and are able to release IL-4, IL-5 and IL13 [13, 17]. IL-13 is known to augment the intestinal permeability and promote enterocyte differentiation and apoptosis [19-21].

Th17 cells may participate in the eradication of extracellular bacteria and fungi [13, 22]. This subset is induced by IL-6 and transforming growth factor (TGF)-β and produces IL-17A, IL-17F, IL-21 and IL-22 [13, 23]. IL-17A attracts neutrophils to the inflammatory site and stimulates the production of pro-inflammatory molecules [13, 24]. Nonetheless IL-17a may also have tissue protective effects in the gut [13, 25].

Equally important are regulatory T-cells (Tregs) who are able to stop Th0 proliferation [26]. They are essential for immune homeostasis by producing

anti-inflammatory cytokines, such as IL10 and TGF-β, and by prohibiting the activation and function of T-cells that are not sufficiently tolerant [13].

In UC higher levels of IL-5 and IL-13 were measured compared to CD and controls, whereas in CD higher levels of IFN-γ were detected [13, 27-32]. Therefore, CD is associated with a Th1-mediated immune response and UC rather with a Th2-associated immune response. In both diseases, high transcript levels of IL-17A were identified in intestinal mucosa, which shows proof of an involvement of Th17 cells [33-35]. Fukaura et al. even observed a higher risk of relapse in UC patients when Th17 related cytokines are increased in colon tissue [36]. Furthermore, in patients with active IBD, Tregs are met in a low frequency in the peripheral blood compared to patients with inactive IBD or controls [13, 37]. However, Tregs with intact function seem to be increased in the intestinal mucosa of IBD patients [37-39]. Although it has also been observed that effector T-cells in the intestinal mucosa of IBD patients are irresponsive to Tregs [40].

As described above, the inflammatory pathway of ulcerative colitis is very complex. The interaction between different cell types and cytokines are not fully understood yet. Our research group took on the approach to design a disease map of UC, which delineates inflammatory processes in UC [41]. However, further studies are required to gain better insight into the inflammatory processes to develop individualized and phase dependent therapeutic regimes.

1.9 Aims of this study

Ulcerative colitis is a very complex disease where the immunological pathways are still not fully understood. The majority of published research on inflammatory bowel disease focuses on one pathway or on one specific cell type and its associated pathways. We took a more comprehensive approach to understand the dynamics of this inflammatory process. Firstly, we determined immunological profiles of patients to identify immune cells that account for the current inflammatory status. Secondly, considering the

heterogeneity of the clinical status in ulcerative colitis patients, we aimed to find a prognostic marker in the peripheral blood that can predict an upcoming inflammation in UC donors. In order to achieve a better understanding, we characterized peripheral blood mononuclear cells (PBMC) with 17 cell surface molecules of Non-UC and UC donors to establish an immunological profile of the disease.

1.10 Working hypotheses

1. Is there a significant difference measurable in the 17 cell surface molecules between Non-UC and UC donors with fluorescence-activated cytometric analysis?
   1.1. We expect an increase of Th2 cells in UC donors compared to Non-UC donors.
   1.2. We expect a decrease of regulatory T-cells in UC donors compared to Non-UC donors.
   1.3. We expect elevated levels of cells of the innate immunity, such as dendritic cells, monocytes and macrophages, in UC donors compared to Non-UC donors.
2. Does Fluorescence-activated cell sorting (FACS) identify a biomarker to predict an upcoming inflammation in UC patients?
3. Does the medication of UC patients influence the immune phenotype?
   3.1. We expect Mesalazine to have an effect on several inflammatory cells, although it is questionable if a systemic effect is to be seen if the drug is applied as a topic formulation.
   3.2. We expect glucocorticoids to have a very broad effect on the immune system; therefore, all cell types could be affected.
   3.3. We expect Azathioprine to have an effect on T-cells and B-cells.
   3.4. We expect TNFα-inhibitors to have a negative influence on innate immune cells.
   3.5. We expect patients after colectomy to show a normal immune profile. According to studies, the quality of life in colectomised patients is

almost as good as the general population [10, 11]. Therefore, we believe in a healthy adaption of the immune system after colectomy.

2 Material & Methods

2.1 Non-UC and UC donors

Twenty millilitres of blood were obtained from the arm vein in trisodium citrate solution (S-Monovette, Sarstedt, Nürnberg, Germany) from 24 Non-UC and 39 UC donors. The control group did not state any history of infectious diseases or symptoms of infection but was not serologically tested. Therefore, they will be referred to as Non-UC donors. All UC donors completed the simple clinical colitis activity index (SCCAI) to evaluate their clinical disease manifestation (see Table 6) [42] . A score ≥ 5 was defined as relapse [43]. The use of the SCCAI was more efficient for us, as no blood tests are needed, and patients can evaluate themselves. Furthermore, a questionnaire designed by our research group was also completed, to get further details of the disease progression over time (see in appendix). The donor characteristics are listed in Table 7. The blood samples were used for FACS analysis.

Table 6: Simple clinical colitis activity index [42]

Symptom	Score
Bowel frequency (day)	
1-3	0
4-6	1
7-9	2
>9	3
Bowel frequency (night)	
1-3	1
4-6	2
Urgency of defecation	
Hurry	1
Immediately	2
Incontinence	3
Blood in stool	
Trace	1
Occasionally frank	2
Usually frank	3
General well being	
Very well	0
Slightly below par	1
Poor	2
Very poor	3
Terrible	4
Extracolonic features	1 per manifestation

Table 7: Donor characteristics

Donor characteristics	UC (n=39)	Non-UC (n=24)
Age (years)		
Mean (SD)	40,4 (16,1)	27,71 (8,4)
Range	19-73	24-59
Sex, male, n (%)	17 (43,59)	6 (25)
SCCAI ≥ 5	11	
Treatment		
Mesalazine	21	
Glucocorticoids	9	
Azathioprine	6	
TNFα-inhibitors	19	
Vedolizumab	2	
Colectomy	9	
None	6	

2.2 Isolation of human PBMC

10 ml of peripheral blood in trisodium citrate solution were diluted with 20 ml of Hank's balanced salt solution (Thermofisher, Waltham, USA). This solution was slowly loaded onto a Leukosep tube (Greiner Bio One, Frickenhausen, Germany) and centrifugated with 800g for 30 minutes. The interphase was separated, diluted with 40 ml Hank's balanced salt solution again and centrifugated for 5 minutes with 1400g. The cell pellet was resuspended in phosphate buffered saline (PBS). [44]

2.3 Fluorescence activated cell sorting

Our staining method is described in Table 8. All antibodies were purchased from Biolegend (San Diego, USA) and applied according to manufacturer's instructions. Samples were processed with a BD FACS CANTO II™ and analysed with FlowJo 10.1-Software (FlowJo LLC, Oregon, USA). The gating strategy is attached in the appendix. [44]

Table 8: Markers used for identification of immune cells in FACS analysis

Cell surface molecule	Identified cell	Reference
CD19+	B-cell	
CD19+ CD27+	Memory B-cell	[45]
CD19+ CD38+	Plasma cell	[45]
CD4+		
CD4+ CD25+	Activated CD4+ T-cell, regulatory T-cell, Th2 cell	[46, 47]
CD4+ CRTH2+	Th2 subset of CD4+ T-cells	[48, 49]
CD8+		
CD14+	Monocyte	[50]
CD14+ CD86+	Mature CD14+ cell	[50-52]
CD14+ CCR2+	Tissue penetrating, inflammatory CD14+ cell	[53]
CD16+	Monocyte	[50]
CD16+ CD86+	Mature CD16+ cell	
CD16+ CCR2+	Inflammatory CD16+ cell	[54]
CD11b+	Classical dendritic cell (cDC)	[50]
CD11b+ CD1a+	CD1a expressing cDC	
CD11b+ CD86+	Mature cDC	
CD11c+	Dendritic cell	[55]

2.4 Statistical analysis

All statistical analyses were performed with R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria (3.2.2). URL https://www.R-project.org/. All values were calculated with the independent samples t-test.

3 Results

3.1 Overview of all FACS results (see Table 9)

Table 9: Overview of all FACS results

	UC/ Non UC		TNFα-inhibitors/ all other UC		Glucocorticoids/ all other UC		Mesalazine/ all other UC		Azathioprine/ all other UC		Colectomy/ all other UC		Colectomy/ Non UC	
Leukocytes (% FoP)	**Δ**	**p-value**	**Δ**	**p-value**	**Δ**	**p-value**	**Δ**	**p-value**	**Δ**	**p-value**	**Δ**	**p-value**	**Δ**	**p-value**
CD4+	**-12,64**	1,57E-05	3,05	0,461	-4,49	0,336	-7,58	0,058	**13,33**	0,020	-9,61	0,053	**-19,59**	0,001
CD4+ CD25+	**-8,11**	5,97E-06	-2,54	0,196	-0,21	0,881	-1,74	0,421	0,06	0,966	3,92	0,372	-5,23	0,256
CD4+ CRTH2+	**0,72**	0,008	-0,62	0,191	1,60	0,091	0,28	0,570	-0,36	0,428	0,60	0,485	1,19	0,176
CD8+	-0,07	0,975	-5,24	0,116	0,75	0,820	0,95	0,783	**10,90**	0,031	-2,71	0,475	-1,93	0,584
CD19+	**-10,24**	0,037	-11,22	0,081	**24,19**	0,042	-2,93	0,660	29,54	0,054	5,21	0,591	-5,90	0,544
CD19+ CD38+	**-34,77**	0,000	**-34,98**	4,84E-05	11,26	0,162	-4,68	0,622	28,92	0,080	11,65	0,254	-25,29	0,030
CD19+ CD27+	1,86	0,574	-2,97	0,570	0,26	0,972	-2,58	0,634	**25,38**	0,003	-5,76	0,429	-2,52	0,716
CD16+	**-4,01**	0,002	1,29	0,326	-0,17	0,893	-0,90	0,504	-3,35	0,079	-1,02	0,469	**-4,79**	0,004
CD16+ CD86+	4,70	0,334	-11,93	0,126	7,53	0,486	-11,53	0,155	17,27	0,289	-0,27	0,981	4,85	0,656
CD16+ CCR2+	-0,53	0,876	-3,30	0,396	-2,75	0,297	3,21	0,387	12,10	0,379	-3,56	0,186	-3,19	0,268
CD14+	0,07	0,954	0,03	0,989	2,00	0,581	-0,31	0,869	-1,55	0,447	3,47	0,348	2,78	0,447
CD14+ CD86+	**-24,65**	0,031	9,95	0,378	-24,56	0,059	-5,65	0,620	-23,92	0,056	-20,78	0,085	**-41,62**	0,004
CD14+ CCR2+	3,36	0,700	-5,63	0,493	**15,58**	0,006	1,97	0,812	0,01	0,999	-0,38	0,974	2,81	0,832
CD11b+	**-6,02**	0,001	-2,47	0,283	2,52	0,532	-2,46	0,283	-1,93	0,464	6,80	0,130	-0,79	0,855
CD11b+ CD1a+	**18,20**	0,002	5,55	0,480	-8,94	0,359	5,92	0,456	-2,58	0,875	-0,94	0,932	17,58	0,125
CD11b+ CD86+	2,46	0,675	**-20,76**	0,011	13,54	0,343	0,79	0,925	25,06	0,248	0,92	0,912	3,46	0,657
CD11c+	**-0,38**	0,035	-0,38	0,090	-0,27	0,271	**-0,52**	0,030	0,27	0,392	0,07	0,840	-0,33	0,385

3.2 Immunophenotyping of UC and Non-UC donors

As shown in Figure 1, UC patients had lower frequencies of CD11b+ cells, CD11c+ dendritic cells, CD16+ cells, CD19+ B-cells and CD4+ T-cells. Furthermore, the subsets of the leukocytes were analysed and showed an increase of CD11b+ CD1a+ cells, CD11b+ CD86+ cells, CD14+ CCR2+ cells, CD16+ CD86+ cells, CD19+ CD27+ memory B-Cells and CD4+ CRTH2+ T-cells in UC patients (Fig. 2). There is also a notable decrease of CD14+ CD86+ cells, CD19+ CD38+ plasma cells and CD4+ CD25+ regulatory T-cells in UC patients compared to Non-UC donors (Fig. 2).

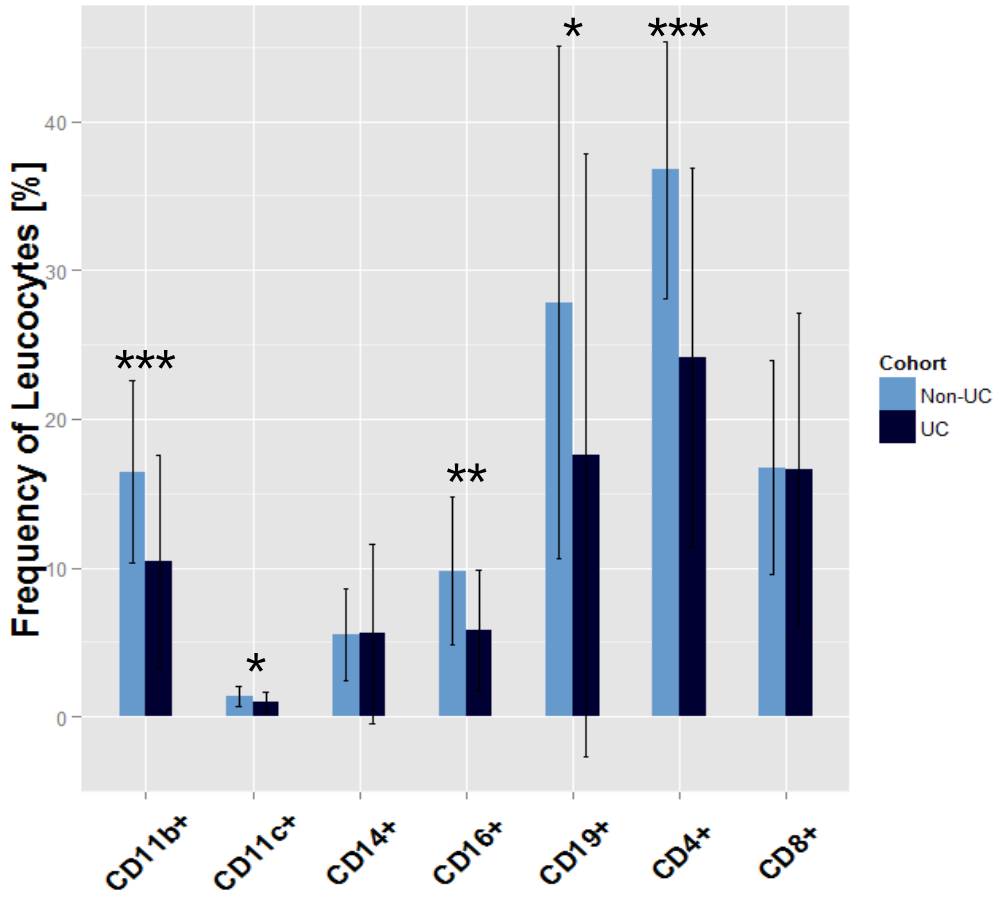

Figure 1: Bar plot depiction of the FACS analysis comparing frequency of human leukocytes isolated from peripheral blood in UC (n=39) and Non-UC (n=24) donors

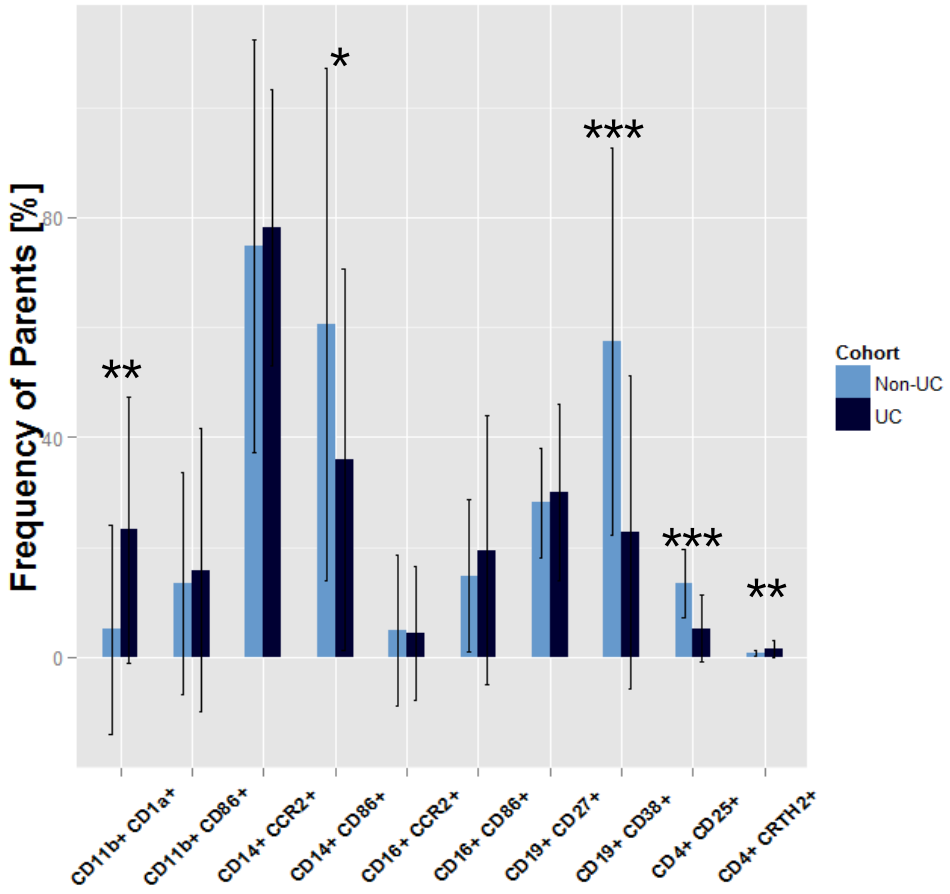

Figure 2: Bar plot depiction of the FACS analysis comparing frequency of subtypes of human leukocytes isolated from peripheral blood in UC (n=39) and Non-UC (n=24) donors

3.3 Effects of treatment in UC donors

As most patients were not therapeutically naïve, one could not conclude from this analysis whether the observed effects were evoked by the inflammation or the result of treatment. Therefore, it is of utmost importance to differentiate between disease-related effects and therapy effects. Since each immune modulatory treatment considered in this analysis has a different mechanism of action, it is possible to compare the effect of the drugs applied. As a result, we were able to compare the UC patients who were administered the specific drug, or who underwent colectomy, to all other UC patients.

3.3.1 Effect of Mesalazine

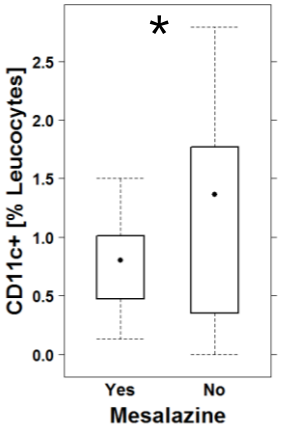

Figure 3: Boxplot depiction of the effect of Mesalazine on CD11c+ cells, yes (n=21), no (n=19)

Patients treated with Mesalazine show significantly lower levels of CD11c+ dendritic cells (Fig. 3).

3.3.2 Effects of glucocorticoids

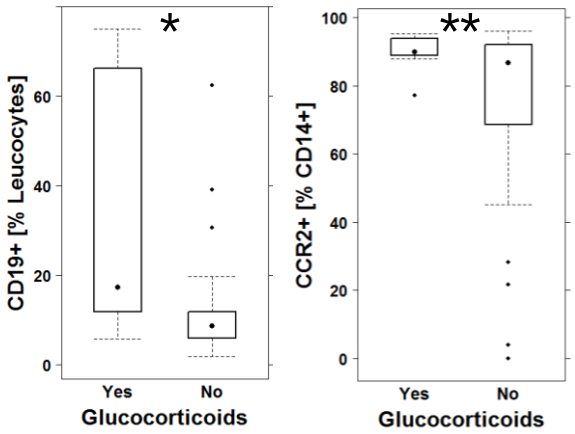

Figure 4: Boxplot depiction of the effects of Glucocorticoids on CD19+ B-cells and CCR2+ CD14+ cells, yes (n=9), no (n=30)

Glucocorticoids lead to an increase of CD19+ B-cells and CCR2+ CD14+ cells in peripheral blood (Fig. 4).

3.3.3 Effects of Azathioprine

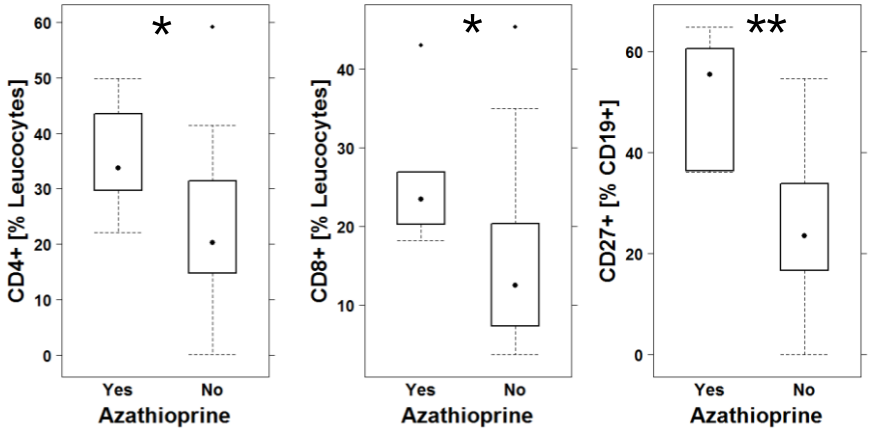

Figure 5: Boxplot depiction of the effects of Azathioprine on CD4+ T-cells, CD8+ T-cells and CD27+ memory B-cells, yes (n=6), no (n=33)

Azathioprine shows a significant increase of CD4+ T-cells, CD8+ T-cells and CD27+ memory B-cells, (Fig. 5).

3.3.4 Effects of TNFα-inhibitors

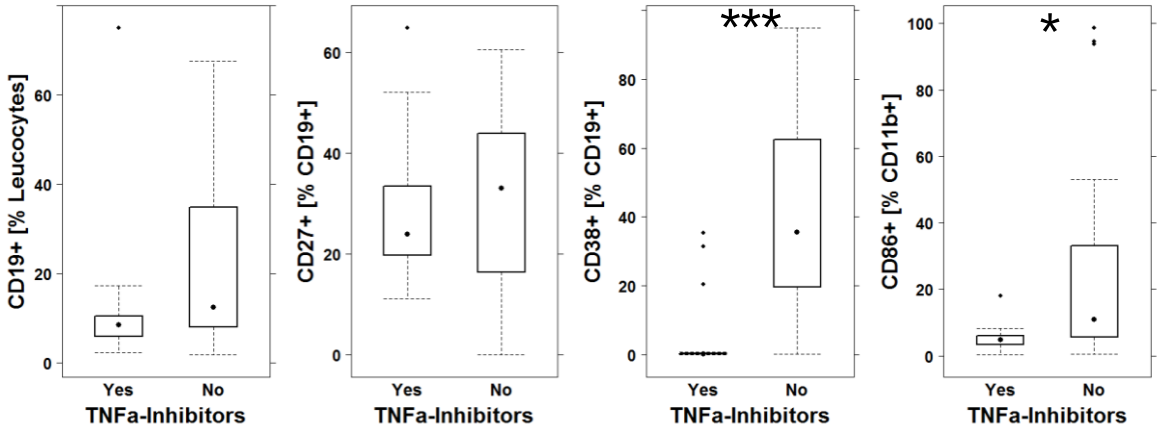

Figure 6: Boxplot depiction of the effects of TNFα-inhibitors on CD19+ B-cells, CD27+ memory B-cells, CD38+ plasma cells and CD86+ CD11b+ cells, yes (n=19), no (n=20)

TNFα-inhibitors cause a decrease of CD19+ B-cells, CD27+ memory B-cells, CD38+ plasma cells and mature CD11b+ cells (Fig. 6).

3.3.5 Effects of colectomy

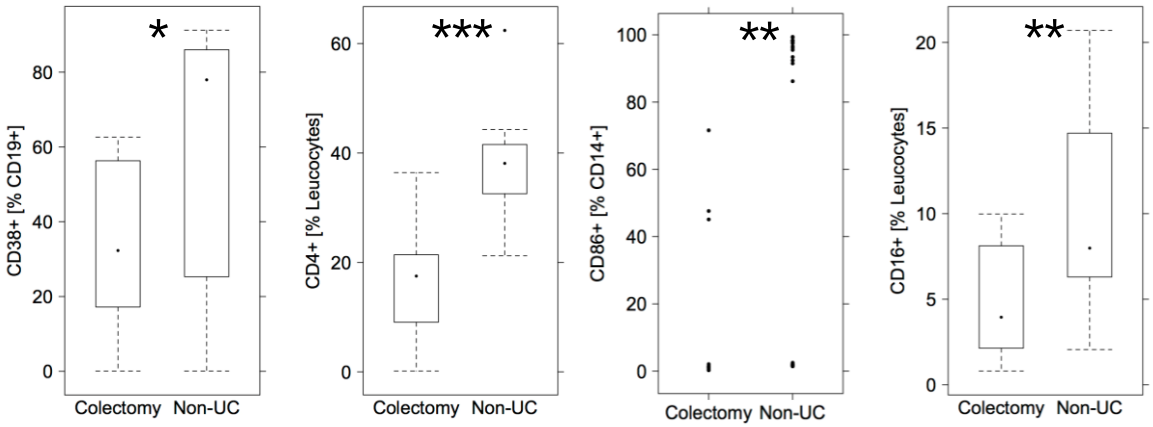

Figure 7: Boxplot depiction of the effects of colectomy on CD38+ plasma cells, CD4+ T-cells, mature CD14+ cells and CD16+ cells, colectomy (n=9), Non-UC (n=24)

In this patient cohort 9 out of 39 underwent colectomy. When compared to the other UC patients, no significant differences in their immunological profile were measurable. If compared to Non-UC donors, plasma cells, CD4+ T-cells, mature CD14+ cells and CD16+ cells were significantly lower in colectomised patients (Fig. 7).

4 Discussion

4.1 Discussion of the working hypotheses

4.1.1 Hypotheses 1 - 1.1-1.3.

A significant difference is measurable with FACS analysis between Non-UC and UC donors. CD4+ CRTH2+ cells, CD4+ CD25+ cells, CD11b+ cells and CD11b+ CD1a+ cells seem to discriminate between UC and Non-UC donors.

As expected, CD4+ CRTH2+ cells are significantly increased in our patient collective, which is not surprising as UC is associated with a Th2 immune response [31].

CD25+ is a marker that can be found on regulatory T-cells (Tregs). It does not proof the regulatory activity of T-cells but the decrease in UC patients suggests a deregulation of the homeostasis of T-cells [56]. Takahashi et al. has previously described this effect in UC patients as well and showed that especially in active UC the decrease of Tregs is associated with an enhancement of colonic inflammation [57].

In contrast to our expectations, cells of the innate immune system are mostly decreased in our patient collective, which could be interpreted as an emigration into the colon. Although if characterized further, CD1a+ CD11b+ cells are more present in the UC cohort compared to Non-UC donors. CD1a+ is a marker that is found on human epidermal Langerhans cells and presents lipids to activate T-cells [58, 59]. The increase of CD1a+ CD11b+ cells in patients with ulcerative colitis has not been described prior to Föhlinger et. al [44]. CD1a+ seems to play a proinflammatory role in a network of many other actors [60]. To determine the exact role of this pathway in ulcerative colitis further analysis is required.

Due to the heterogeneity of the UC patient collective, as well as the possible influences of therapy upon them, it is beneficial to create subgroups according to their specific medications. This allows us to distinguish the effects of therapy from disease specific characteristics.

4.1.2 Hypothesis 2

At this point it is not possible to discriminate a biomarker for disease progression. Unfortunately, the patient population was very heterogeneous in regard of their medication and clinical appearance. Therefore, it is quite difficult to isolate single effects.

4.1.3 Hypotheses 3 – 3.11-3.5.

The medication of UC patients influences the immune phenotype as it leads to an immune suppression.

Effect of Mesalazine

We observed significantly lower levels of CD11c+ cells in patients treated with Mesalazine. Although this effect is questionable as the drug was applied topically in most cases.

Effects of glucocorticoids

In patients receiving treatment with glucocorticoids, an increase of B-cells has been observed. This effect has been previously described as a temporary effect in the beginning of treatment with these agents [61]. If glucocorticoids are given systemically for a longer period than two weeks a decline in B-cells has been described which might be a delayed onset of the drug effect [61]. In our patient collective the glucocorticoids were applied rectally and/or systemically. The duration of application varied by patient.

Effects of Azathioprine

It has been described that Azathioprine leads to apoptosis of T-cells and can also prevent activation and proliferation [62]. In our UC cohort, CD4+ T-cells, CD8+ T-cells and CD27+ B-cells are measured in higher quantities than in Non-UC donors. Therefore, it could be interpreted as a loss of effect in our UC patient cohort.

Effects of TNFα-inhibitors

A decline of CD11b+ CD86+ cells has been observed in this patient cohort. A study in the United Kingdom has identified the same effect in patients with rheumatoid arthritis [63]. After treatment with TNFα-inhibitors the CD80+ and

CD86+ levels decreased, showing a disruption in maturation [63]. Furthermore, the stimulation of T-cells by DC's was impaired [63]. In conclusion, TNFα-inhibitors disrupt an important interaction between innate and adaptive immune system leading to a poor T-cell stimulation [63].

In addition, our patient collective who were treated with TNFα-inhibitors, we observed a decrease of plasma cells (p= 4.837e-05) and CD27+ memory B-cells (not significant) when compared to patients with no TNFα blockade. Salinas et al. showed that a 12-week TNFα blockade of PBMCs collected from spondyloarthritis patients did not impair differentiation of B-cells into plasma cells in vitro [64]. They also observed an increase of CD19+ CD27+ cells after twelve weeks of TNFα blockade [64]. In contrast, Anolik et al. witnessed lower frequencies of CD27+ memory B cells in patients with rheumatoid arthritis [65]. We can therefore assume that our observation could be an effect of the TNFα-inhibitors in patients with ulcerative colitis, or that it could be attributed to the fact that these patients were treated longer with TNFα-inhibitors and most of them were concurrently treated with multiple immune modulatory drugs.

A recent study by Jodeleit et al. suggests the existence of different inflammatory dynamics in UC and supposedly patients with monocyte driven inflammatory phases do not respond well to treatment with adalimumab [66]. This data shows how important it is to understand the complex mechanisms in UC in order to select the right therapy to reduce the rate of non-responders and side effects.

Effects of colectomy

There were no significant differences in the immunological profile in patients who underwent colectomy compared to patients who didn't. On the other hand, there was a measurable decline, if compared to Non-UC donors concerning CD4+ T-cells, plasma cells, mature CD14+ cells and CD16+ cells. This implies that even after removing the main inflammatory site, they do not regain a healthy immunological profile. This might also explain why patients suffering from ulcerative colitis have a higher risk of pouchitis than patients with familial adenomatous polyposis (FAP), even though the same surgical procedure is performed [67, 68]. In a retrospective analysis 23,3% of UC patients suffered from chronic pouchitis after IPAA procedure [7].

The decrease of plasma cells was also observed as a therapy effect from TNFα-inhibitors. Most patients feel an improvement of their clinical status after colectomy, therefore implying that a decline of plasma cells in peripheral blood can be related to the disease activity in the colon.

4.2 Strengths and limitations of this study

One of the strengths of this study is the use of peripheral blood mononuclear cells. It is cost efficient, more accessible and less invasive than retrieving colon tissue. However, it is unclear if the distribution of the inflammatory cells in peripheral blood is comparable to that in the human colon. In our research group this correlation was conducted on inflamed colons of patients who underwent colectomy. This analysis showed that CD1a+ CD11b+ cells and NK-T cells were the main actors of the local inflammation [44].

The major limitation concerns the number of patients and Non-UC donors included in this study, especially taking into consideration that UC may be an umbrella diagnosis covering different pathological phenotypes. In addition, this patient cohort was very heterogeneous in regard of their treatment. Only six patients were currently not taking any medications, therefore, we do not have a proper insight in therapy naïve patients or patients at the time of primary diagnosis. There is also a selection bias considering that most patients treated in our hospital had severe symptoms that required a complex therapy. In spite of this bias, this analysis does include potential therapy effects in ulcerative colitis, which allow conclusions to be drawn about relevant pathways in UC.

5 Conclusion

The immune profile of UC patients was distinct from that of Non-UC donors. CD25+ CD4+ cells, CD4+ CRTH2+ cells, CD11b+ cells and CD1a+ CD11b+ cells were identified as biological markers to discriminate between Non-UC and UC donors. The immune profile of colectomised patients was similar to that of other UC patients, indicating that the removal of the main targeted organ does not restore a healthy immune system. This might explain the predisposition of colectomised UC patients to develop a pouchitis. The results from this study corroborate the hypothesis that a comprehensive approach might lead to a better understanding of the immunological processes underlying the pathology of UC. However, future studies will have to be improved regarding subtypes of immune cells and must include the analysis of cytokines and the histologic evaluation of colon tissue. In conclusion, immunological profiling can help us understand the complex mechanisms underlying ulcerative colitis. This can further lead to the identification of more specific targets for drugs and consequently a better and personalized treatment.

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Appendix

Patient questionnaire for ulcerative colitis

Date:	
Age?	
Sex?	
Age at the time of diagnosis?	
Height?	
Weight?	
Abdominal pain?	O yes O no
If yes, where?	O Upper abdomen: O Middle abdomen O Lower abdomen Oright Omiddle Oleft
Abdominal cramps?	O yes O no
Time of pain	
Everyday?	O yes O no
At night?	O yes O no
Diarrhoea?	O yes O no ____ per day ____ per night O bloody O mucous?

Are you currently receiving any treatment?	O yes O no
If yes, which medications are you taking?	
Is the disease under good control with the medication?	
Form of disease activity?	O chronic intermittent (relapses + full remission) O chronic-continuous (symptoms of variable intensity, no full remission) O acute fulminant (toxic megacolon)
How often do you experience symptoms?	O daily O weekly O monthly O yearly
How long were you free of symptoms before this relapse?	
How do you notice the beginning of a relapse?	
Do you feel that you can influence the frequency of a relapse? (without medication)	
General symptoms?	
Fever?	O yes O no
Joint pain?	O yes O no

Back pain?	O yes O no
Skin lesions?	O yes O no
Eye infection?	O yes O no
Fatigue?	
Circulatory complaints?	O yes O no
Psychological distress?	O yes O no
	O yes O no
Primary sclerosing cholangitis?	O yes O no
Weight fluctuation?	O weight gain? ____kg O weight loss? ____kg O unchanged
Previous operations on the bowel?	O yes O no
If yes, which?	
Are any members of your family affected from ulcerative colitis?	O yes O no
Are any of your family members affected from the following diseases?	O Allergies? O Asthma? O Skin diseases?

Gating strategy

The following figure shows the gating strategy for the identification of human leukocytes and subtypes. First, human leukocytes were gated according to forward scatter and side scatter. The application of specific antibodies enabled the characterization of each cell type (see figure 8 below).

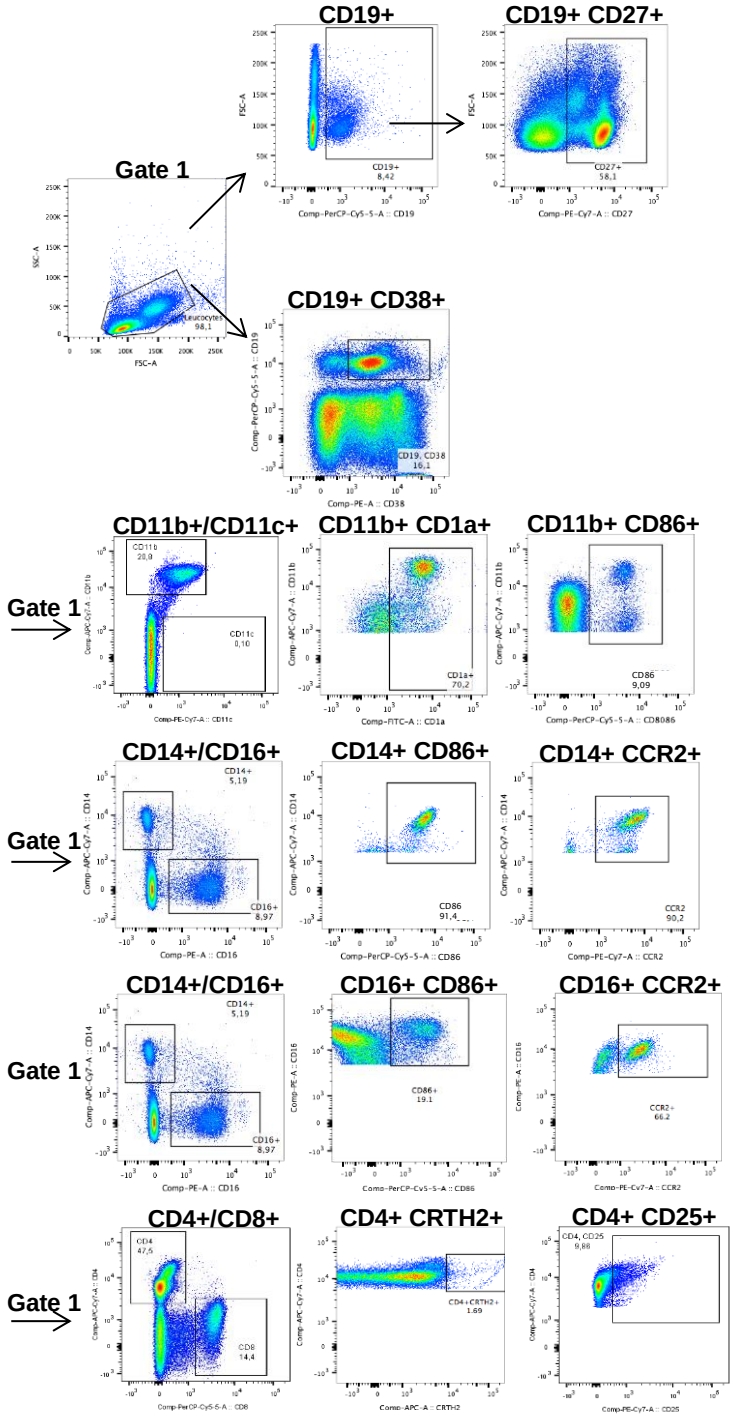

Figure 8: Gating strategy for the identification of human leukocytes and subtype

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