DISSERTATION

The impact of anti-α4β7-Integrin therapy on T cells and clinical parameters in Inflammatory Bowel Disease: Exploring potential therapy outcome predictors

Der Einfluss der anti-α4β7-Integrin Therapie auf T-Zellen und klinische Werte in chronisch entzündlicher Darmerkrankung: Die Suche möglicher Therapieprädiktoren

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Preface

Konstantin Fritz has contributed with the described work as an author to the following publication:

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The first four authors marked with a star shared the first authorship.

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Abbreviations

CCL25	C-C motif chemokine ligand 25
CCR	Chemokine receptor
CD *(followed by a number)*	Cluster of differentiation
CD *(followed by no number)*	Crohn´s disease
CrP	C-reactive protein
CTLA4	Cytotoxic- T lymphocyte-associated protein 4
EMA	European Medicines Agency
ELISA	Enzyme-linked-Immunosorbent-Assay
FACS	Flow cytometry
GPR15	G Protein-coupled receptor 15
HD	Healthy donor
HLADR	Human Leukocyte Antigen – DR isotype
IBD	Inflammatory Bowel Disease
Ig	Immunoglobulin
IL	Interleukin
JAK	Janus kinase
Ki67	Kiel 67
MAdCAM-1	Mucosal addressin cell adhesion molecule 1
MCH	Mean corpuscular haemoglobin
MCV	Mean single volume of erythrocytes
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PD1	Programmed cell death protein 1
ROC-Curve	Receiver operating characteristic curve
T_{CM}	Central memory T cells
T_{EM}	Effector memory T cells
T_{EMRA}	Effector memory T cell re-expressing CD45RA
T_{regs}	Regulatory T cells
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TNF	Tumour necrosis factor
UC	Ulcerative colitis
VCAM-1	Vascular cell adhesion molecule-1

0. Abstract

0.1 Abstract in English

Introduction: The underlying mechanisms of Inflammatory Bowel Diseases (IBD) are still under investigation. Accordingly, therapy is so far limited to block the overreaction of the immune system in the gut. A new attempt is to block immune cells from entering the gut, which is used in the biological vedolizumab (VDZ). However, real-life studies show a high non-response rate. Therefore, predictive markers are needed. But results so far have not demonstrated enough significance yet.

Aims of study: This study investigated the influence of VDZ – especially in T cell populations - and searched for predictive markers for therapy response.

Methods: 50 patients were included in this study between July 2018 and November 2021 by receiving open-label VDZ. A balanced, healthy control group was collected as well.

The clinical data of patients was monitored during the treatment. If patients reached at least a three-point reduction in the Harvey-Bradshaw-Index and at least a two-point reduction in the Partial Mayo Score by week 30, they were defined in retrospect as responders. Stool samples were collected at week 0, 6 and 18-22 to measure calprotectin. Blood samples were collected at the same time points to determine VDZ trough level in serum and isolate peripheral blood mononuclear cells (PBMCs) from blood. Those were then analysed by flow cytometry for different T cell subgroups. Clinical and flow cytometry data were examined to show how VDZ influences the immune system during the induction phase. Also, differences between responders and non-responders were investigated. The flow cytometry data were also analysed for differences between healthy donors and IBD patients at baseline.

Results: From the analysed clinical markers, only the inflammatory markers CrP and calprotectin, as well as the haematological markers MCV, MCH and thrombocyte count could predict therapy outcome at week 6. The trough level of VDZ at week 6 did not show any significant difference between responders and non-responders. The analysis of the flow cytometry data showed a lower $CD3^+$ T lymphocyte count in IBD patients compared to healthy donors in the blood. From the T cell subgroups only $CD8^+$ T_{EMRA} show statistically significantly higher levels in IBD patients. The gut-homing markers CCR9 and GPR15 were highly expressed on memory T cells in IBD patients. The marker which showed a significant predictive power for therapy outcome was the expression

of Ki67 on $CD3^+$ T lymphocytes with an 80% sensitivity and 68.42% specificity. Further investigating which T cells express Ki67, $CD4^+$ effector memory T cells turned out to be the subgroup with the highest Ki67-expression. Their co-expression of CD38 and Ki67 also showed a high predictive power for therapy response with 80% sensitivity and 78.95% specificity.
Discussion: Ki67 expression shows to be a predictive marker that needs more investigation as to whether it could be used as a standard clinical test before deciding on using VDZ as a therapy.

0.2 Abstract in German

Einleitung: Die Ursachen für chronisch entzündliche Darmerkrankungen sind bisher noch nicht abschließend geklärt. Deshalb ist das Therapiespektrum darauf begrenzt, den Pathomechanismus zu blockieren. Der neue Aspekt, den Eintritt von Immunzellen in den Darm zu verhindern, wird in dem Biologikum Vedolizumab (VDZ) genutzt. Da es häufig zu einem Therapieversagen kommt, werden prädiktive Marker dringend gesucht. Bisher gibt es keinen mit einer ausreichenden Signifikanz.
Ziel der Studie: Diese Studie untersuchte den Einfluss der Therapie mit VDZ – speziell auf T-Zellpopulationen – und prädiktive Marker für das Therapieansprechen.
Methodik: 50 Patient*innen wurden zwischen Juli 2018 und November 2021 in die Studie eingeschlossen, während sie VDZ erhielten. Gleichzeitig wurde eine ausgewogene, gesunde Kontrollgruppe gesammelt. Die klinischen Parameter der Patient*innen wurden kontrolliert. Sofern Patient*innen mindestens drei Punkte im Harvey-Bradshaw-Index beziehungsweise zwei Punkte im Partial Mayo Score bis zur 30.Therapiewoche verloren hatten, wurden sie retroperspektivisch als Therapieerfolg gewertet. Stuhlproben wurde zu den Zeitpunkten Woche 0, 6 und 18-22 gesammelt, um Calprotectin zu bestimmen. Blutproben wurden an denselben Zeitpunkten abgenommen, um die Konzentration von VDZ im Serum zu bestimmen und PBMCs zu isolieren. Diese wurden mittels Durchflusszytometrie (FACS) auf die verschiedenen T-Zellgruppen untersucht. Die Veränderungen in den klinischen und durchflusszytometrischen Daten wurden auf den Einfluss von VDZ hin untersucht. Auch Marker, die ein Therapieansprechen voraussagen könnten, wurden analysiert. In den FACS-Daten wurden zudem Unterschiede zwischen den gesunden Kontrollen und den Patient*innen vor Therapiebeginn erforscht.
Ergebnisse: Unter den analysierten klinischen Markern konnten zum Zeitpunkt Woche 6 die Werte der Entzündungsmarker CrP und Calprotectin sowie der hämatologischen Marker MCV, MCH und Thrombozytenzahl das Therapieresultat signifikant vorhersagen. Die Konzentration von

VDZ im Blut bei Woche 6 konnte dies nicht signifikant bestimmen. Die Analyse der FACS-Daten zeigten eine niedrigere Lymphozytenzahl im Blut bei Patient*innen im Vergleich zu den Kontrollen. In den T-Zell-Subgruppen zeigten nur $CD8^+$ T_{EMRA} signifikante erhöhte Werte in Patient*innen. Die Antigene CCR9 und GPR15 waren signifikant erhöht auf T-Gedächtniszellen in Patient*innen. Das Antigen, welches Therapieansprechen signifikant vorhersagen konnte, war die Expression von Ki67 auf Lymphozyten mit einer 80% Sensitivität und 68.42% Spezifität. Weitere Untersuchungen zeigten, dass $CD4^+$ Effektor Gedächtniszellen die höchste Ki67-Expression aufwiesen. Die Koexpression von CD38 und Ki67 zeigte auch eine hohe Vorhersagekraft mit einer 80% Sensitivität und 78.95% Spezifität.

Diskussion: Ki67 scheint ein prädiktiver Marker zu sein. Es benötigt weitere Untersuchung, ob seine Expression als praktischer, klinischer Test zur Therapiewahl genutzt werden kann.

1. Introduction

1.1 Inflammatory Bowel Disease

The gut is one of the biggest surfaces in the human body (1). As it is constantly exposed to many antigens, the gut-associated immune system must balance between an adequate reaction to pathogens and harmless antigens (2). This balance is lost in Inflammatory Bowel Diseases (IBD), which are defined as chronic disorders in the gastrointestinal tract (3).

IBD can be subclassified into Crohn´s Disease (CD) and ulcerative colitis (UC). There are several differences between the two: CD mostly appears in the ileocecal region but can also involve any other part of the gastrointestinal tract. It presents itself as segmental inflammation, which involves all bowel layers. This can lead to strictures, fistulae, and abscesses (3). UC involves mostly the rectum and can extend to the whole colon but not the rest of the gastrointestinal tract. The inflammation is limited to the mucosa and usually spreads continuously along the large intestine often resulting in intestinal bleeding (3).

The causes of IBD are still unknown. The hypotheses about its origin scan be separated into two main fields: Genetics and environmental factors (4,5). Several studies searched for a genetic component in IBD. An argument supporting the genetic cause has been given by studies of Brant et al. and Hallfvarson et al: They found a 20 to 50 percent concordance between monozygotic twins and 10 percent in dizygotic twins, of whom one had CD. The concordance values for UC with 15% and 5% indicated a lower heritability (6,7).

A critical factor that explains the IBD pathway is a variation in the NOD2 gene, which could be linked by Ogura et al. to a higher incidence of CD (8). NOD2 encodes for a protein that activates NF-κB, which in turn correlates with higher responsiveness to bacterial lipopolysaccharides. Other genome-wide association studies identified another 200 risk loci essential for immune response, cell stress regulation, epithelial barrier function, and microbial defence (9–11as cited in 12). However, a single pathogenic axis could not be described so far, which leads to the conclusion that IBD is a polygenic-caused disease.

The environment can influence the gut barrier as well: Epidemiological studies could link smoking as a risk factor for Crohn´s Disease (13). Furthermore, a diet with a high uptake in “milk protein, animal protein and polyunsaturated fatty acids [may] increase the risk for IBD” (14 as cited in 15). The most discussed hypothesis is the so-called hygiene hypothesis, which proposes that as we get less exposure to pathogens in modern times, our immune system loses the ability to train which

results in an overreaction seen as an autoimmune disease – such as IBD but for example also asthma (3). This would imply that the shift into our industrialized society is linked to the rise in IBD: Changes in our diet, the use of disinfectants and antibiotics but also the urbanization of society, which exposes us to higher air pollution, can be correlated with IBD (16). This hypothesis is supported by the fact that IBD is more prevalent in industrial countries (17). "The highest annual incidence of ulcerative colitis was found in Europe with 24,3 per 100.000 persons-years for UC and CD with 20.2 per 100.000 in North America. Also, in the prevalence North America (UC, 249 per 100,000 people; CD, 319 per 100,000 people) and Europe (UC, 505 per 100,000 people; CD, 322 per 100,000 people) are leading." (18). As economic progress happens in developing countries, the gap to the industrial nations becomes smaller (18).

Another rising field of interest to explain IBD lies in microbiota research. Its composition is very heterogeneous in-between humans as it can be influenced by genetics and external factors (16). But its influence on IBD risk could be shown by some studies: For example, a less diverse microbiome with a higher portion of pro-inflammatory bacteria such as *Enterobacteriaceae* and *Fusobacteriae* could be linked to a higher IBD prevalence (19–21 as cited in 22).

All these influences result in a weakened intestinal barrier, causing a massive leukocyte infiltration to attack intruding pathogens. As the developing tissue damage weakens the intestinal barrier even more, a vicious circle begins to unfold, which leads to the full picture of the disease.

To diagnose IBD, a combination of clinical markers, radiological and invasive imaging is needed (23): The best tool for the correct diagnosis is a colonoscopy and/or gastroscopy with the taking of histological biopsies to clearly identify IBD. Several markers such as C-reactive protein (CrP) and leukocyte count are used to monitor the inflammation level. In addition, measuring faecal calprotectin, which can be found in neutrophils (24), points to an increased intestinal inflammation.

1.2 Overview of therapy options to treat IBD

Even though the pathogenesis of IBD is not fully understood, some therapy options have been developed. Corticosteroids are a group of drugs which are frequently used in the treatment of many autoimmune and autoinflammatory diseases and have proven effective in IBD as well, especially in rapidly reducing the disease activity highly inflamed patients. Nevertheless, corticosteroids are not used for long-term therapy due to their associated side effects (25).

Another group of medication is formed by anti-metabolites like azathioprine and methotrexate. Those can be employed for keeping up the remission of inflammation but should also not be used

long-term because of the high possibility of adverse events such as myelosuppression. A French study even reported a higher prevalence of lymphoproliferative disorders in IBD patients receiving azathioprine (26). Besides these groups, there are several drugs which can be used as comedication or in mild disease such as aminosalicylates and calcineurin inhibitors.

However, given all these drug groups have limited efficacy and several adverse events associated with their use (27) other options were needed and developed. The idea was to specifically target certain parts of the IBD pathway. Thereby, not the whole immune system would be blocked which could result in immunosuppression together with several side effects. Instead, only specific overreacting pathways should be targeted. A big leap was the introduction of anti-TNFα-antibodies like infliximab and adalimumab. They made it possible to treat patients who were refractory to conventional therapy (28). Their mechanism is to neutralize tumour necrosis factor α (TNFα) thereby preventing the interaction with its receptor (29). Its result is the induction of T cell apoptosis and the Fc-receptor-dependent promotion of wound-healing macrophages (30 as cited in 31).

The effectiveness of these anti-TNFα-antibodies could be proven in clinical trial studies: "69 percent of patients (with ulcerative colitis) receiving 5 mg [per kg body weight] of infliximab and 61 percent of those who received 10mg [per kg body weight] had a clinical response at week 8" (32) - measured by a reduction in their disease activity index. In Crohn's disease patients the response numbers were even higher, reaching 82% (33). For adalimumab the remission rate was 16.5% at week 8 in UC patients (34) and 79% to 83% of CD patients (35).

Putting their effectiveness aside, these drugs can still cause immunosuppression resulting in opportunistic infections like tuberculosis (36). Also, paradox immunological reactions in the joints and the skin have been reported (37), which can lead to a termination of the therapy.

Taking the clinical trial results into a real-life perspective shows a poorer result, as well: Long-term studies showed that 10% to 13% of patients lose therapy response per year during anti-TNFα treatment (38–40 as cited in 37). Qiu et al. even demonstrated in a meta-analysis loss of response in 33% of patients taking infliximab and 41% of patients with adalimumab in the first year (41 as cited in 29).

Hence other targets directed against the inflammatory pathway had to be searched for: A promising example is ustekinumab, which is neutralizing the Interleukin-12p40 (IL-12p40) subunit shared by IL-12 and IL-23 (42 as cited in 31). Also, Janus kinase (JAK) inhibitors such as filgotinib, which influences the signalling of IL-6, IL-10 and the Interferon family, show clinical benefits in

Crohn´s disease patients (43 as cited in 31). This suggests that targeting multiple cytokine pathways could be a promising therapy scheme (31).
Another idea is to shift the focus from inhibiting different complicated signalling pathways to just blocking immune cells from getting into the gut in the first place.

1.3 Gut-homing of immune cells

To be able to enter the gut, "naïve T cells circulate through secondary lymphoid organs until they encounter their cognate antigen presented by retinoic acid-producing dendritic cells in the gut-associated lymphoid tissue" (44). Thereby, the cells do not only get activated and proliferate but also are imprinted for gut-homing (44). "T cells imprinted for small intestinal homing express integrin α4β7, α4β1, β2 integrins and CCR9, while cells primed for migration to the colon show high levels of integrin α4β7 and GPR15." (44)
These integrins are transmembrane cell adhesion receptors. They always consist of a non-covalently associated heterodimer (45) between an α- and a β-chain. 18 α- and 8 β-subunits have been identified so far which can be combined in 24 different heterodimers leading to different functions (27). One prominent example is α4β1-integrin, which facilitates the migration into the central nervous system, bone marrow, and skin by binding to vascular cell adhesion molecule-1 (VCAM-1) (46–48 as cited in 27). The combination, which is used to enter the gut, is α4β7-integrin. These are the only two known combinations of α4-integrin. β7-integrin in return can also bind to αE-integrin. This heterodimer is expressed on dendritic cells (49 as cited in 45), as well as a few circulating blood lymphocytes and lymphocytes located in the lamina propria (45). But almost all intraepithelial lymphocytes express this integrin combination (45), leading to the conclusion that it leads to the retention of lymphocytes in the gut epithelium through binding to E-cadherin (50 as cited in 45).
α4β7-integrin itself is highly expressed "on IgA-secreting plasma cells, memory T cells and activated gut-homing $CD4^+$ T cells" (51 as cited in 45) but in low levels on naïve T cells and on B cells (52 as cited in 45). This can probably be explained as naïve T cells have not yet been imprinted for intestinal homing (53,54 as cited in 55). Furthermore, "[natural killer] (NK) cells, activated monocytes, macrophages, eosinophils and [dendritic cells]" express α4β7-integrin (56,57 as cited in 45).
The main receptor for α4β7-integrin is mucosal addressin cell adhesion molecule 1, short MAdCAM-1 (58 as cited in 45). "MAdCAM-1 is constitutively expressed on high endothelial

venules of both mesenteric lymph nodes and Peyer´s patches as well as on postcapillary venules of the lamina propria of the small and large intestine.“ (45). The other receptor binding to α4β7-integrin is vascular cell adhesion molecule-1 (VCAM-1) (45), which is mostly expressed on endothelial cells (59). Nevertheless, for the interaction between VCAM-1 and α4β7-integrin a higher state of activation is needed compared to MAdCAM-1 (58 as cited in 45), as well as another costimulatory molecule (60).

The interaction of α4β7-integrin and MAdCAM-1 is part of a multistep process starting with the T cells´ migration to the gut along chemotactic gradients (see Figure 1). On the endothelial wall of the blood vessels integrins and selectins (mostly L-selectin) start loosely binding to their ligands resulting in the cells slowing down and rolling along the endothelial wall (60 as cited in 44). This increases the possibility that tissue-secreted chemokines like C-C motif chemokine ligand 25 (CCL25) activate the cells causing a conformational change of the integrins (61 as cited in 60). This way they can now bind firmly to MAdCAM-1 and migrate through the endothelium into the tissue.

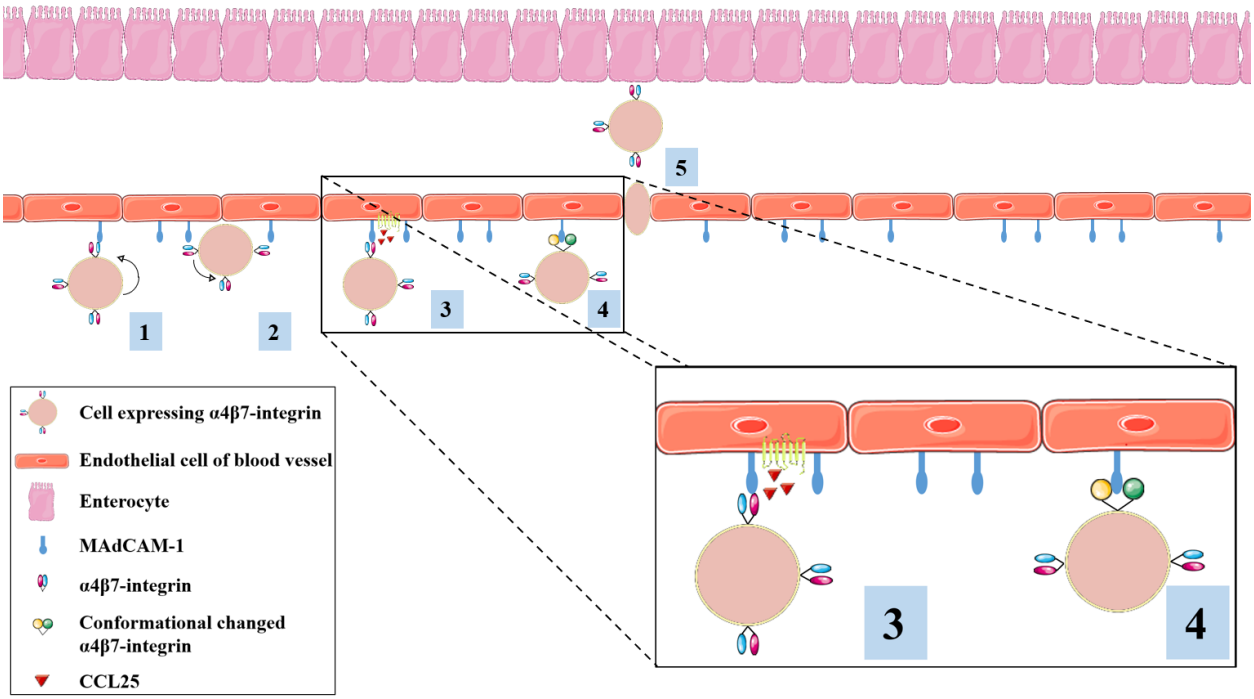


Figure 1: Gut-homing of immune cells via α4β7-integrin

(Modified from Figure 1 "Targeting Immune Cell Trafficking–Insights from Research Models and Implications for Future IBD Therapy" (44)*; the image was drawn using pictures created by Servier Medical Art)*

1. *Immune cells start loosely binding to their ligands, which initiates a rolling process*
2. *The rolling process slows the cells down*
3. *CCL25 interacts with α4β7-integrin*
4. *A conformational change happens in α4β7-integrin resulting in a firm binding to MAdCAM-1*
5. *The cells can now enter the gut through the endothelial wall*

Boden et al. could find that the expression of MAdCAM-1 is elevated in inflamed tissues in IBD patients (55) which underlines the key role of this homing process for the autoimmune process in IBD.

That is why this gut-homing mechanism was selected as a target for another biological drug: vedolizumab.

1.4 Vedolizumab: Mode of action and clinical trials

The first substance to block integrin interaction was natalizumab which is used in the treatment of multiple sclerosis. It blocks the α4-integrin in both α4β1- and α4β7-integrin (62). Therefore, it was

also included in the therapy of IBD patients. However, a higher prevalence of progressive multifocal leukoencephalopathy (PML), a rare but often fatal opportunistic infection caused by the JC virus, was observed in treated IBD patients (27).

Thus, its use in IBD was discontinued and a new antibody targeting the more gut-specific integrins was created: Vedolizumab, "a recombinant humanized immunoglobulin G1 (IgG1) produced in Chinese hamster ovary cells was generated by fusing the binding domains from the mouse anti-human α4β7-integrin monoclonal antibody Act-1 to a conventional human IgG1 scaffold." (63 as cited in 64). To eliminate cytotoxic Fc-mediated effects, two mutations were introduced into the Fc region of vedolizumab (65 as cited in 64). Vedolizumab binds specifically to α4β7-integrin to block its interaction with MAdCAM-1 (66 as cited in 64).

This was tested in clinical trials: Patients with UC had a response rate of 47.1% at week 6 compared to 25.5%. "At week 52, 41.8% of patients who continued to receive vedolizumab every 8 weeks were in clinical remission (Mayo Clinic score ≤ 2 and no subscore ≥ 1), as compared with 15.9% of patients who switched to placebo" (67). The remission numbers were only slightly higher for a therapy pattern of receiving vedolizumab every 4 weeks with 44.8% (67). No higher incidence of side effects was reported in the patients´ group compared to placebo. In 3.7% of patients´ antibodies against vedolizumab were produced (67).

CD patients had lower numbers: "A total of 14.5% of patients (…), who received vedolizumab and 6.8% who received placebo were in clinical remission."(68). 39.0% and 36.4% of patients, who were then assigned to receive vedolizumab every 8 weeks and every 4 weeks, were in clinical remission at week 52, compared to 21.6% in the placebo group (68). In addition, patients with Crohn´s Disease experienced more side effects than patients with ulcerative colitis and with 4.1% also had a higher rate of patients who produced antibodies against vedolizumab (68).

Because of its effectiveness and safety, vedolizumab was approved under the commercial drug name "Entyvio" for the treatment of adult patients with moderately to severely active ulcerative colitis or Crohn´s Disease. It is administered as a 300mg intravenous infusion over approximately 30 minutes at 0, 2 and 6 weeks and every 8 weeks thereafter "(64). Its advantage over many other biological drugs is that it can be given subcutaneously via pen as well, which gives the patients more freedom, as they do not have to come to a healthcare facility for their next infusion.

1.5 Vedolizumab: Real-life studies

Real-life studies showed a mixed picture compared to the clinical trials: Christensen et al. reported that 31% of CD patients and 35% of UC patients reached remission at week 54 – which is lower than in the clinical trials (69). A study by Amiot et. al. also showed with 27.2% lower numbers for CD patients at week 54 but comparable numbers of patients with UC (40.5%) (70). These results are comparable to the studies by Kotze et al.: In their study, 22.1% of CD patients reached clinical remission, whereas 61.9% of UC patients reached it by week 54 (71). These findings underline – similarly to the clinical trials – a better response in UC to vedolizumab treatment than in CD. However, they also show that only less than half of all patients reach clinical remission after one year of treatment.

This calls for markers which could predict therapy response beforehand to identify patients who benefit from vedolizumab versus patients who will experience treatment failure. So far, several ideas have been investigated.

1.6 Clinical markers for therapy response

Many clinical markers have been tested to predict therapy response to vedolizumab: For example, basic markers like gender have not been shown to be predictive (55,72). One study reported an association between colonic localisation and better responses to vedolizumab which has not been confirmed in other studies (73 as cited in 74). As vedolizumab is often used after failure of anti-TNFα-therapy, many studies also analysed whether there is a difference in therapy outcome between anti-TNFα-naïve and –experienced patients: The GEMINI studies could already find a significant difference between these two groups regarding therapy response (67,68), which could be shown in other studies as well (75). However, Allegretti et al. found no significant differences among patients who had received prior treatment with more than one biological drug (72). In addition, some papers discussed disease activity as a possible predictor. No IBD-related hospitalisation in the past 12 months (75) and a low score in the Harvey-Bradshaw-Index (76) could be shown to be predictive of clinical remission in CD patients. Other inflammation markers such as IL-6, IL-8 and TNFα were also increased in non-responders in several studies (77,78). CrP is still under investigation as a predictive marker showing differing results: While some studies found it statistically significantly higher in non-responders (72), this could not be proven in others (55). A further commonly used inflammation marker is calprotectin, which was identified to be statistically significantly different at week 6 for UC, but not CD (78).

Another idea to show predictive power was to analyse the therapy management: For example, adding another immunomodulatory drug to the therapy regimen in addition to vedolizumab increased therapy response in CD patients in one study (72), even though there have been also contradicting reports (79). However, the main focus in this field is monitoring the trough level of vedolizumab itself: A lot of studies found a significant difference between responders and non-responders, displaying higher levels in responders (80,81). However, the cut-off level to differentiate the two groups varies in some studies: Most set it at 22-24 mg/ml at week 6 of the therapy (82,83). But Ungar et al. found the median level of vedolizumab to be at 29.7mg/ml in patients with active disease (84). This shows that the trough level measurement is promising but needs further study.

Overall, clinical markers are not yet conclusive, as studies contradict each other's findings or they show predictive power in only one of the two IBD diseases.

1.7 Further markers for therapy response

Therefore, another attempt to find a predictive marker was on the genetic level: Zeissig et al. could show that vedolizumab intervention led to the downregulation of inflammatory gene expression in innate immune cells like monocytes in both UC and CD patients who achieved remission (85). Verstockt et al. could even developed a model of four genes (RGS13, DCHS2, MAATS1 and PIWIL1) to predict endoscopic remission with an accuracy of 80% (86), even though their link to vedolizumab treatment is not fully understood yet. Also, the level of exosomes specifically binding vedolizumab has been discussed to influence therapy outcomes (87). However, genetic testing is still expensive for clinical routine.

Ananthakrishnan et al. also proposed microbiota to be a predictive field: They identified that "[co]mmunity α-diversity […], *Roseburia inulinivorans* and a *Burkolderiales* species were more abundant at baseline among (Crohn´s disease) patients achieving week 14 remission" (22). Further research in that area is needed.

The main field of interest has so far been to understand the interaction of vedolizumab and its target α4β7-integrin. Several studies found that responders had a higher α4β7-integrin-expression on multiple T cell subsets before therapy (29,55,88). However, a more recently published study found the opposite (89).

Not only the level of α4β7-integrin-expression has been under discussion for its predictive power but also the cell subsets that express it or get impacted by vedolizumab treatment. While most

studies show the T cells to play a key role (90), others pointed out the importance of the innate immune cells in vedolizumab treatment outcome: Kim et al. could show in a retrospective study with 251 IBD patients that a higher mean eosinophil count at baseline in gut tissue was predictive of a vedolizumab therapy response (5). Other studies also found an influence of vedolizumab treatment on the innate immune system (85,91). Unfortunately, its interaction with vedolizumab and predictive power for therapy outcome has yet to be discovered.

1.8 Aims of the study

The effect of vedolizumab on the different immune cell subsets has not been completely understood yet. Most studies suggest the main targets are T cells, but this still needs clarification. Moreover, the therapy response of vedolizumab is as low as of other biological drugs in IBD therapy. Furthermore, vedolizumab has another disadvantage in comparison to the other antibody-based biologicals: As it is blocking inflammatory cells from entering the gut, rather than downregulating the inflammation process itself, its clinical effects take longer to work compared to the anti-TNFα-drugs (92). However, it is a major downside for patients to wait up to 14 weeks to know whether vedolizumab is working for them as a good medication. Therefore, good prediction markers are needed to identify patients who might benefit from vedolizumab before starting the therapy. Even though many studies tried to find a good marker, there has been none so far which has a high predictive power or can be easily integrated into the clinical work.

Therefore, my thesis aims to answer the following questions:

1. Is there a marker –clinical or immunological – which can predict therapy response to vedolizumab before starting treatment?
2. How does vedolizumab influence the immune system, especially T cells and their subsets?

2. Materials

2.1 Disposable materials

Consumables	Source (Identifier)
CrytoPure tubes	Sarstedt (65.386.007)
Eppendorf tubes	Sarstedt (72.706)
FACS tubes	Sarstedt (55.1579)
Filter mesh (width 10)	Sefar Nitex (03-80/29)
Heparin blood tubes	Greiner (455084)
MicroAmp Clear Adhesive Film	ThermoFisher Scientific (4306311)
PCR Plate, 96 well plate, low profile	ThermoFisher Scientific (AB0800)
Serum blood tubes	BD (367953)
50ml tubes	Sarstedt (62.547.254)
96-well plate, U-shaped	Greiner (650 180)
96-well plate, V-shaped	Sigma (82.1583)

2.2 Reagents and Kits

Reagents	Source (Identifier)
Ammonium chloride (NH_4Cl)	PanReac AppliChem (141121.1211)
BD FACS Lysing Solution 20x Concentrate	BD (349202)
Bovine Serum Albumin, Fraction V, PAN Bio	Pan Biotech (P06-1391500)
Brefeldin A	Cayman (Cay11861-25)
Compensation Beads, Anti-Rat Ig	BD (552844)
Compensation Beads, Anti-Rat and Anti-Hamster Ig	BD (552845)
Compensation Beads, Anti-Mouse Ig	BD (552843)
Dimethyl sulfoxide	Sigma-Aldrich (276855-250ML)
DNase I, Lyo., 100 MG	Roche (11284932001)
Entyvio 300mg	Takeda (EU/1/14/923/001)
Ethylenediaminetetraacetic acid (EDTA)	Carl Roth (Art.-Nr. 8040.3
FcR Blocking Reagent	Miltenyi Biotec (130-059-91)
Fetal Bovine Serum	Corning (Ref: 35-079-CV)

Foxp3/Transcription Factor Staining Buffer Set	Invitrogen (00-5523-00)
HEPES	Pan Biotech (P05-01100)
Hydrochloric acid (HCl)	Carl Roth (Art.-Nr. 4625.1)
IgG Mouse (polyclonal)-unconjugated	Dianova (015-000-003)
Ionomycin	Sigma (I0634-1MG)
Pancoll human, Density: 1.077g/ml	Pan Bio Tech (P04-60500)
Phorbol-12-myristat-13-acetat (PMA)	Sigma (P1585-1MG)
Phosphate buffered saline (PBS)	Lonza (882104-12)
Penicillin-Streptomycin (10,000 U/mL)-10	Gibco (15140122)
Potassiumhydrogencarbonate ($KHCO_3$)	PanReac AppliChem (141480.1211)
Precision Count Beads	Biolegend (424902)
Proteomic Stabilizer	Fisher Scientific (501351691)
RPMI Medium 1640 (1X) +GlutaMAX ™-I	Gibco (Ref 61870-010)
Saponin purified from Quillaja Bark	Sigma (S4521-25G)
Trypan blue Solution (0.4%)	ThermoFisher Scientific (15250061)
Vedolizumab drug level ELISA	Immundiagnostik (REF: K9658)
10% Na-azide solution (sterile filtered)	Sigma Aldrich (S2002-100MG)
2-Mercaptoethanol (50 mM)-20 mL	Gibco (31350-010)

2.3 Media buffers and solutions

ACK Lysing Solution: 4.15g NH_4Cl+0.5g $KHCO_3$+18.5mg Na_2EDTA+400ml H_2O; adjust pH to 7.2-7.4 with 1 HCl and fill up to 500ml with H_2O

Brefeldin A: Stock conc. 5mg/ml in DMSO, conc. For assay 5µg/ml

cRPMI: RPMI + GlutaMAX + 1% 2-Mercaptoethanol + 1% P/S + 10% FCS

FACS buffer: 1x PBS, 0.05% BSA, 0.01% NaN_3, 2 mM EDTA

Freezing medium: 90% Sterilized fetal bovine serum + 10% DMSO

PMA/Ionomycin: PMA: Stock conc. 500µg/ml in DMSO, conc. For assay 5ng/ml; Ionomycin: Stock conc. 500µg/ml in DMSO, conc. For assay 500ng/ml

Saponin: 0.05% Saponin in FACS Buffer

Thawing medium: RPMI + 20% FCS + 10mM HEPES + 1X P/S

Trypan blue: 10% of Trypan blue 0.4% solution + 90% PBS+0.01%Azid

2.4 Antibodies

Fixable Viability Dye: Dilute 100 tests in 300µl sterile PBS

Compensation Beads, Anti-Rat and Anti-Hamster: 6ml anti-rat/hamster + 6ml negative beads + 38ml PBS/BSA/AZID

Compensation Beads, Anti-Mouse Ig: 6ml anti-mouse + 6ml negative beads + 38ml PBS/BSA/AZID

Marker	**Conjugate**	**Species**	**Dilution**	**Company**	**Clone**	**Ordering number**	**Lot number**
CD49d (Integrin α4)	BV786	Human	1:200	Biolegend	9F10	304344	B283212
CCR4	BV750	Human	1:50	BD	1G1	746980	1315811
Integrin β7	BV711	Human/ Mouse	1:800	Biolegend	FIB504	321239	B312129
CD25	BV650	Human	1:200	Biolegend	BC96	302634	B334256
Ki67	BV551	Human	1:800	Biolegend	Ki67	350517	B326873
CCR6	BV605	Human	1:100	Biolegend	G034E3	353420	B291940
HLADR	BV570	Human	1:200	Biolegend	L243	307637	B314476
CD161	BV421	Human	1:50	Biolegend	HP-3G10	339914	B334269
Fixable Viability Dye	eFluor 780	Human	1:800	ThermoFisher		65-0865-14	
CD127	AlexaFluor 700	Human	1:100	Biolegend	A019D5	351344	B239351
GPR15	APC	Human	1:100	Biolegend	SA302A10	373006	B315684

CXCR3	PerCP-Cy5.5	Human	1:100	BD	1C6/CXCR3	560832	1165016
PD1	AlexaFluor 488	Human	1:250	Biolegend	EH12.2H7	329935	B303068
TIGIT	PE/Cy7	Human	1:600	Biolegend	A15153G	372713	B288970
CD29 (=β1)	PE/Cy5	Human	1:1500	Biolegend	TS2/16	303005	B321101
CCR9	PE/Dazzle 594	Human	1:100	BD	11F2	358917	B303105
CTLA4	PE	Human	1:100	Biolegend	BNI3	369603	B340713
CD103 (=αE)	BUV805	Human	1:800	BD	Ber-ACT8	748501	1189085
CCR7	BUV737	Human	1:75	Thermofisher	3D12	741786	1015473
CD45RA	BUV661	Human	1:1500	BD	5H9	741654	1189043
CD38	BUV615	Human	1:400	BD	HIT2	751138	1189092
CD8	BUV563	Human	1:1500	BD	RPA-T8	612915	1014122
CD3	BUV496	Human	1:100	BD	UCHT1	317332	1125207
CD4	BUV395	Human	1:300	BD	RPA-T4	564724	1207421
CD45RA	PE-Cy7	Mouse	1:300	Biolegend	HI100	304126	B314937
CD3	PerCP-Cy5.5	Mouse	1:300	Biolegend	OKT3	317336	B280475
Integrin β7	FITC	Rat	1:200	Biolegend	FIB504	321214	B242741

Vedolizumab	Cy5	Human	1:400	Purchased from Takeda and conjugated by Deutsches Rheuma Forschung szentrum	EU/1/14/923/001		
CD4	Pacific Orange	Mouse	1:500	Produced by Deutesches Rheuma Forschung szentrum	Production date: 21.07.2015		
CD49d (Integrin α4)	BV421	Mouse	1:400	Biolegend	9F10	304322	B248598

Table 1: Antibodies used in all experiments

Antibodies marked in red were only used in the experiment, in which α4β7-detection was analysed, while unmarked vedolizumab bound to it

2.5 Equipment

BD FACS Canto™ II Clinical Flow Cytometry System

https://www.bdbiosciences.com/en-eu/products/instruments/flow-cytometers/clinical-cell-analyzers/facscanto

BD FACS Symphony™ A5 Cell Analyzer

https://www.bdbiosciences.com/en-us/products/instruments/flow-cytometers/research-cell-analyzers/bd-facsymphony-a5

Infinite F50 Tecan

https://lifesciences.tecan.de/products/microplate_readers/infinite_f50

2.6 Software

BD FACSDiva Software (Version 6.1.3); BD (https://www.bdbiosciences.com/en-ca/products/instruments/software-informatics/instrument-software/bd-facsdiva-software-v-6-1-3-upgrade-kit.643630)

FlowJo (Version 10.5.3); FlowJo (https://docs.flowjo.com/flowjo/getting-acquainted/10-5-release-notes/10-5-3-release-notes/)

Graphpad (Version 9.3.1 (471)); Graphpad (https://www.graphpad.com/updates/prism-931-release-notes)

Magellan™ (Version 7.0)

Tecan https://lifesciences.tecan.com/software-magellan

3. Methods

3.1 Patient cohort

IBD patients, who got induced with vedolizumab, were recruited in the outpatient clinic and ward at the Benjamin-Franklin-Campus of the Charité clinic between July 2018 and November 2021. The only exclusion criteria were not being able to speak German, not being of legal age and/or not being able to consent themselves. Patients were informed before and signed a consent form (IBDome-study; EA4/162/17).

The open-labelled treatment pattern, which the patients then received afterwards, can be divided into an induction phase and a maintenance phase: The induction phase is six weeks long, during which the patients receive three infusions of each 300mg vedolizumab at weeks 0, 2 and 6. In the maintenance phase after that the patients received vedolizumab every eight weeks either via a subcutaneous pen or infusion.

The patients were monitored during their therapy, especially in the first 30 weeks: First of all, a patient´s status was examined through a standard questionnaire for each UC (Partial Mayo Clinical Score, see Table 2) and CD (Harvey-Bradshaw-Index, see Table 3).

Symptom	**Severity**	**Score**
Stool frequency per day	Normal	0
	1-2 stools more than normal	1
	3-4 stools more than normal	2
	≥ 5 stools more than normal	3
Rectal bleeding	None	0
	Blood in <50% of stool	1
	Clear blood in stool	2
	Blood without stool	3
General assessment	Normal	0
	Mild disease	1
	Moderate disease	2

	Severe disease	3
Complications	Arthralgia, Uveitis, Erythema nodosum, Aphthous ulcers, Pyoderma gangrenosum, Anal fissure, new fistula, Abscess, axial arthritis, peripheral arthritis	1 point each

Interpretation

Partial Mayo Score	**Severity**
<2	Remission
2-4	Mild disease
5-6	Moderate disease
>6	Severe disease

Table 2: Partial Mayo Score

Standard clinical examiner to assess disease activity in UC patients

Symptom	**Severity**	**Score**
General wellbeing	Well	0
	Impaired	1
	Poor	2
	Very poor	3
	Unbearable	4
Abdominal pain	None	0
	Mild	1
	Moderate	2
	Severe	3

Number of liquid stools per day		1 point each
Abdominal resistance	None	0
	Unclear	1
	Definite	2
	Definite and painful	3
Complications	Arthralgia, Uveitis, Erythema nodosum, Aphthous ulcers, Pyoderma gangrenosum, Anal fissure, new fistula, Abscess, axial arthritis, peripheral arthritis	1 point each

Interpretation

Harvey-Bradshaw-Index	**Severity**
<5	Remission
5-7	Mild disease
8-16	Moderate disease
>16	Severe disease

Table 3: Harvey-Bradshaw-Index

Standard clinical examiner to assess disease activity in CD patients

Before every vedolizumab infusion 36ml blood in heparin tubes and 9ml in serum tubes were collected at weeks 0, 2 and 6 and continued at the infusion between week 18 and 22 of the treatment plan. To determine their response to the therapy, patients were not only asked the medical questionnaire, but stool samples were collected at weeks 0, 6 and 22, in which the calprotectin level was measured (see Figure 2).

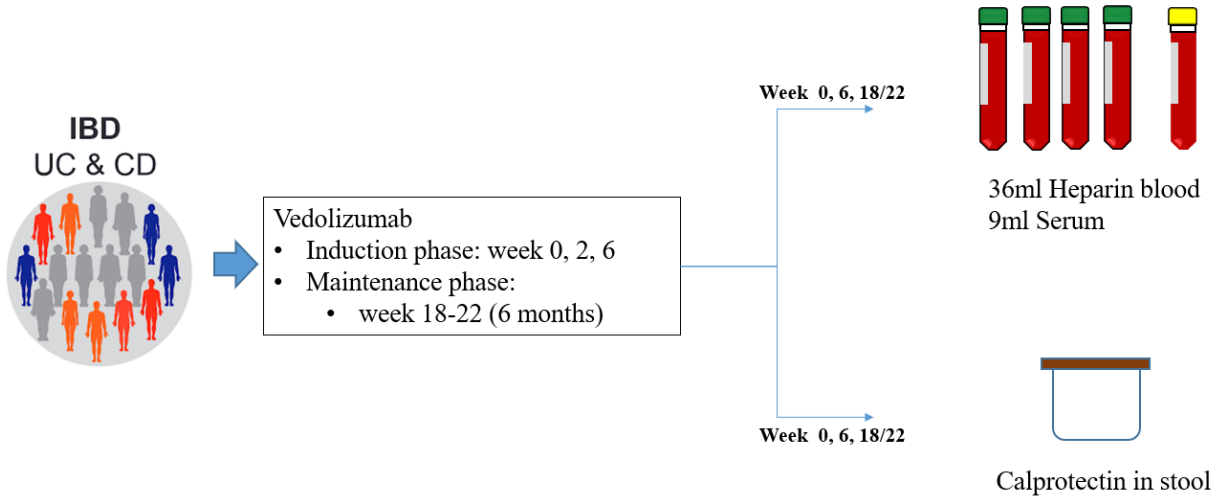


Figure 2: Study design

Patients get monitored during the induction phase of therapy (week 0-6) and have a follow-up during the maintenance phase. At each timepoint blood and stool samples are taken.

Therapy response was defined as a reduction of at least three points in the Harvey-Bradshaw-Index and at least two points in the Partial Mayo Score by week 30, as it has been used in many studies so far (93). Other factors which supported this decision were remission shown in endoscopy or a reduction in the calprotectin level. If the patients did not reach these criteria by week 30 or had a therapy failure, they were categorized as non-responders. I did not include patients in the response analysis who had low levels in their clinical scores and in inflammation marker levels throughout the whole therapy as I classified them as staying in remission, in which the level stayed the same. To get an overview of the immunological changes, several standard laboratory markers such as CrP, leukocytes count, thrombocytes, lymphocytes, monocytes, as well as neutrophile, eosinophile and basophile granulocytes were searched for in the clinical histories in retrospect. Also, other basic parameters like erythrocyte count, haemoglobin, mean single volume of erythrocytes (MCV) and mean corpuscular haemoglobin (MCH) were documented to examine a possible blood formation disorder linked to a reduction in iron uptake over the gut.

3.2 Blood Processing

3.2.1 Serum Isolation

To isolate serum out of the collected blood a protocol was established by my group. After the anonymisation of the samples, the serum tube was processed first by centrifuging it at 2000 g for 7 minutes at room temperature (see Figure 3). Afterwards, the serum supernatant was isolated and 3ml were divided into two 1.5ml Eppendorf tubes (Sarstedt (72.706)). These were labelled and stored away at -80°C.

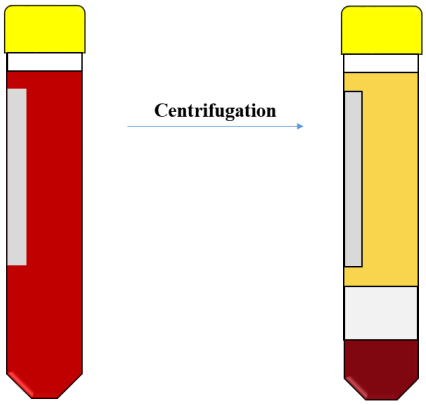


Figure 3: Serum isolation

3.2.2 PBMC isolation

Next, the content of the heparin tubes was mixed into one 50ml tube. The heparin tubes were washed out with PBS (Lonza (882104-12)) and this was added to the rest of the whole blood in the 50ml tube. PBS was added until the tube contained 50ml. Two new 50ml tubes (Sarstedt (62.547.254)) were taken out. In each 15ml of Pancoll (Pan Bio Tech (P04-60500)) were added. On top of that, 25ml of the blood-PBS mixture was layered so that the two would not mix. The two tubes were centrifuged at 800xg for 20 minutes at room temperature with an acceleration of 4 and a deceleration of 1.

After the centrifugation, the following components were found in the following order from top to bottom (see Figure 4): Blood plasma and PBS, Peripheral Blood Mononuclear Cells (PBMCs), Pancoll and at the bottom erythrocytes pellet. The blood plasma was sucked away carefully, so the PBMCs could be taken up into a new 50ml tube. The rest was discarded. The PBMCs were diluted in 50ml PBS. This was centrifuged at 350xg for 10 minutes at 4°C, as also unwanted parts could have been transferred into the new tube. If there were, for example, erythrocytes as a red pellet

still visible, the cells were then diluted in 10ml of Ammonium-Chloride-Potassium Lysing buffer – an Erythrocyte lysis buffer - (preparation: see Materials). They were then left in the buffer for two minutes at room temperature. Afterwards, the whole mixture was diluted in PBS up to 50ml and then centrifuged again at 350xg for 10 minutes at 4°C.
The supernatant was discarded, and the pellet was diluted in 10ml PBS.

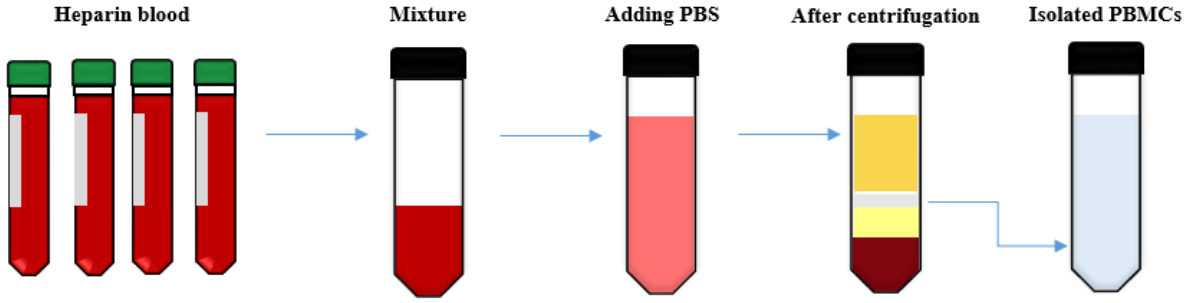


Figure 4: PBMC isolation

10µl out of it were diluted in 90µl of Trypan blue (preparation: see Materials), so the cells could be counted with a Haemocytometer. Afterwards, the cell suspension was centrifuged again at 350xg for 10 minutes at 4°C. The cells were diluted in freezing medium (preparation: see Materials) down to 1x10^7 per millilitre. Then one millilitre each was transferred to a cryotube (Sarstedt (65.386.007)). These were first stored in boxes at -80°C and transferred to a liquid nitrogen tank for long-term storage.

3.3 Vedolizumab trough level measurement through Serum ELISA

The frozen serum samples were thawed on ice for two hours. 200µl of serum was then transferred into a 96-well plate and frozen again. This was done to have several 96-well plates as a backup. For the Enzyme-linked-Immunosorbent-Assay (ELISA) a prepared kit and its protocol were used (Immundiagnostik; (REF: K9658)). Wash buffer concentrate was diluted 1:10 before use. Prepared standards and controls were reconstituted with 500µl of ultrapure water. The conjugate concentrate was diluted 1:101 in wash buffer and frozen again until everything was ready. After all reagents had been prepared, the serum was thawed again and diluted 1:200 in the prepared sample dilution buffer. 100µl of diluted samples, controls and standards were pipetted on their marked wells on the special ELISA well plate, which already contained the bound antibodies against vedolizumab. After that, the plate was covered and incubated for 1 hour at room temperature on a horizontal shaker, which was then turned to 550rpm with an orbit of 2mm. Afterwards, the content was washed five times with each 250µl wash buffer. Next, 100µl of the conjugate was added to each well, followed by incubation for 1 hour at room temperature on the shaker. The washing step was repeated. Next, 100µl of a substrate was pipetted into each well and incubated for 15 minutes at room temperature in the dark. The colour change from blue to yellow was observed and 100µl stop solution was added to each well. The well plate was read out directly afterwards into an ELISA reader. Absorption was determined at 450nm against 620nm as a reference. As an analysis program to visualize the data, Magellan ™ was used.

3.4 Flow cytometry

3.4.1 Analysed surface markers

To analyse the isolated PBMCs, three flow cytometry panels were created by Prof. Dr. Dr. Ahmed Hegazy, Camila Cancino and Dr. Veronika Horn based on some of their previous findings through mass spectrometry that some $CD4^+$ T cell clusters seemed to be predictive for the therapy outcome of IBD patients treated with vedolizumab. Flow cytometry experiments should validate these findings.

In this thesis, the surface marker panel will be discussed (see Figure 5): First, since α4β7-integrin – the target of vedolizumab – is mostly expressed on T cells, the panel was focused on T cells.

That is why, CD3 was included as a marker. CD4 and CD8 were also added into the antibody panel to differentiate the two subgroups of T cells: Helper T cells, which express CD4, and Cytotoxic T cells with their defining expression of CD8. By including CD45RA and Chemokine receptor 7 (CCR7) as antibodies in the panel, they can be further subdivided into memory T cells, which had contact with a pathogenic antigen, and naïve T cells without an antigen contact yet. They can be differentiated, as naïve T cells still express CD45RA, which memory T cells lose. The expression of CCR7 makes it possible to distinguish the two memory T cells groups – central memory T cells (from now on called T_{CM}; $CD45RA^{-}CCR7^{+}$) and effector memory T cells (from now on called T_{EM}; $CD45RA^{-}CCR7^{+}$): Whereas T_{EM} circulate through the bloodstream and can be recruited into inflamed tissue right away, T_{CM} reside in lymphoid organs and can be activated later to clonal expansion for further support (94).

The other T cell subgroups which can also be characterised are regulatory T cells (T_{regs}) for CD4 T cells by gating on $CD25^{+}$-cells. Their function is to balance a homeostasis between the inflammatory response and suppressing it to prevent autoinflammatory reactions (95).

The Cytotoxic T cells do not contain many regulatory T cells, which is why they were not analysed. But a subgroup which can be found in the $CD8^{+}$ T cells are the T effector memory cells re-expressing CD45RA (from now on called T_{EMRA}). They have a high cytotoxicity even without antigen contact (96). The other subgroups of T cells that can be analysed are Th1, Th2 and Th17 cells. However, as the antibody for CXCR3 did not work properly in the analysis, a differentiation into these groups was not possible. All in all, T cell subgroups could clearly be analysed.

The two gut-homing markers CCR9 and G Protein-coupled receptor 15 (GPR15) got added to the mix as well. The main group – the integrins – containing α4, β7, αE and β1 were included to analyse their heterodimer combinations. Furthermore, antigens which showed to have predictive power for therapy response were investigated: Those are CD38 and HLADR. In contrast, markers which reduce T cell activation were also included: Those are PD1, TIGIT and CTLA4. A further gut homing marker – CD161 – was included, as well.

The last antigen to be included was proliferation marker Kiel 67 (= Ki67). It is normally used in the monitoring of cancer forms, as it is a sign of how aggressive the tumour will grow. As it has a key function in the cell cycle (97), we thought it to be a good marker of cell proliferation.

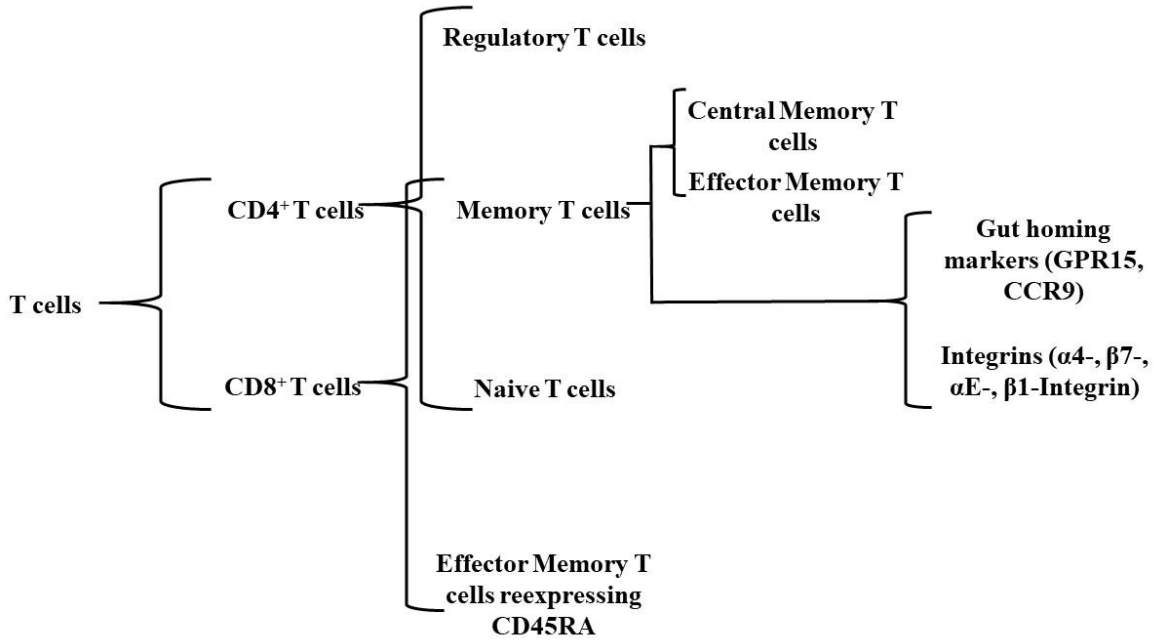


Figure 5: Analysed cell groups

$CD4^+$ and $CD8^+$ T cells and their subgroups were analysed and especially their integrin and gut homing marker expression

3.4.2 Antibody titration

For all these markers corresponding antibodies were checked and titrated before using them on the patients´ samples. Some antibodies which were used had not been established in my laboratory before. Because the utilized concentration was unknown, a titration experiment was done. For that, freshly isolated PBMCs were treated with different dilutions of the antibody (no staining, 1:50, 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200) and a backbone antibody mix, to be able to gate on cell subsets, that express the marker, the antibody is targeted against. The staining process was done according to the protocols which is described in the following chapter. In the analysis, the geometric ratio (the ratio between the geometric mean of the antigen-positive and -negative population) and frequency of parent were shown of the markers. The decision on the right concentration was based on which dilution showed the correct frequency of the marker according to literature and a good geometric ratio between positive and negative populations (see Figure 6). The mixture of all antibodies used in their right dilutions was then tested on healthy patients as well to see that each antigen could be marked correctly in the mixture of all antibodies: For that, the concentration but also the setup of the lasers of the FACS Symphony needed to be adapted.

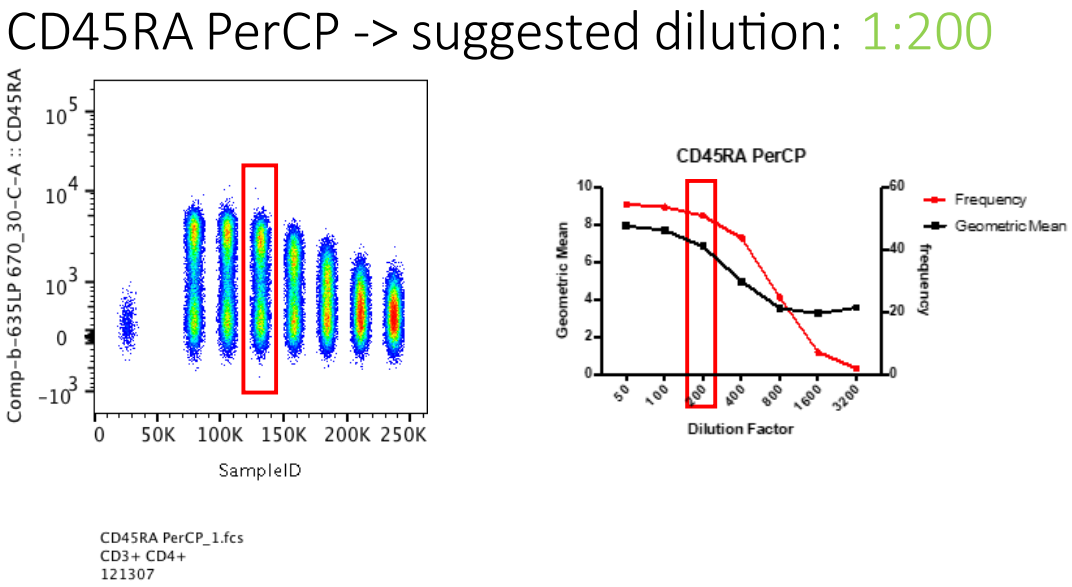


Figure 6: Example of analysis of titration results

A dilution was chosen, at which a clear differentiation between antigen expressing and non-expressing samples is possible, and the geometric mean and frequency are close to what is described in the literature.

3.4.3 Detection of α4β7-integrin-expression with bound vedolizumab

Before analysing the PBMCs of the patient cohort, the measurement of how α4β7-integrin-expression would be influenced by vedolizumabs´ binding was tested.

For that, PBMCs from six healthy controls were isolated. These were split up and stained with either none, 0.1, 1, 10 or 100 µg/ml of unmarked vedolizumab (Takeda (EU/1/14/923/001)), which was previously diluted in FACS Buffer (preparation: see Materials) to the correct dilution (see Table 4).

Dilution (µg/ml)	Added volume from the next higher dilution (µl)	Added FACS buffer (µl)
Stock =1,2mg/ml		
100	49.8	550.2
10	120	540
1	120	540
0.1	120	540

Table 4: Dilution of unlabelled vedolizumab to block α4β7-integrin

All following steps were performed on ice. After leaving the different vedolizumab concentrations for 30 minutes on the cells, the unbound vedolizumab was washed off three times with FACS buffer. Next, a master mix of antibodies to mark CD3, CD4, CD45RA, α4, β7, as well as anti-mouse-IgG and Fixable Viability Dye PE-Cy7 (ThermoFisher; 65-0865-14) - a marker for to gate out dead cells – were added (see Table 5). In addition, vedolizumab which was bound to the Cy5-fluorochrome was stained on the cells. This would bind to the remaining epitopes of α4β7-integrin, which the previously added unlabelled vedolizumab did not yet occupy. The decreasing signal of Cy5 would prove that different concentrations of unmarked vedolizumab had blocked α4β7-integrin before. A control was only stained with Fixable Viability Dye, anti-mouse IgG, CD3-, CD4- and CD45RA-antibodies to be able to properly gate on α4-, β7- and vedolizumab-Cy5 staining.

Marker	Fluorochrome	Dilution	Company	Clone
CD45RA	PE-Cy7	1:300	Biolegend	HI100
CD3	PerCP	1:300	Biolegend	OKT3
β7	FITC	1:200	Biolegend	FIB504
Vedolizumab	Cy5	1:400	Purchased from Takeda and conjugated by DRFZ	EU/1/14/923/001
CD4	Pacific Orange	1:500	Produced by DRFZ	Production date: 21.07.2015
CD49d (=α4)	BV421	1:400	Biolegend	9F10
Fixable Viability Dye	eFluor 780	1:500	ThermoFisher	Ref.: 65-0865-14

Table 5: Antibody panel for experiment to detect α4β7-integrin-expression while being blocked by vedolizumab

All cells were incubated for 15 minutes at 4°C in the dark. After washing them twice again with FACS buffer, BD Lyse and Fix buffer was added (BD (349202)) to fix the antibody staining. The samples were kept for 10 minutes at room temperature in the dark. Next, the cells were washed again with FACS buffer three times.

Finally, the cells were diluted in FACS buffer and acquired at a BD FACSCantoII Clinical Flow Cytometry System. The results were then analysed with FlowJo and visualized in GraphPad Prism.

3.4.4 Staining protocol

After all this preparation, the patients´ PBMCs could be analysed. First, the frozen PBMCs were thawed by transferring them from the liquid nitrogen tank into a 37°C water bath. The cells were quickly thawed and transferred under sterile conditions into a 1ml warm thawing medium (preparation: see Materials). This was then centrifuged at 350xg for 10 minutes at 4°C. After the centrifugation, the supernatant was discarded, and the pellet was resuspended in 5ml of thawing medium. A 10 µl aliquot was diluted in 90 µl Trypan blue. 10 µl out of that mixture were then

counted with a Haemocytometer to calculate the cell number. One million cells were used for the staining with the surface panel.

Afterwards, 100 µl each of the cells were distributed to three wells: After spinning the cells down, one well was stained with the whole master mix of antibodies, whereas the second was only stained with the backbone antibodies and the third was resuspended in just medium, to be able to properly analyse the data: With these two stainings, the signal of all markers could be compared to the well with only medium. The staining for the specific target markers, which were only included in the master mix but not in the backbone (see Table 6) could then be even better differentiated by comparing master mix and backbone. IgG (Dianova (015-000-003)) was added to all in the concentration 1:100 to block the Fc receptor, as well as Fixable Viability Dye (dilution 1:800) to be able to gate out dead cells and DNAse (50 U/ml) so that dead cells could not coagulate with each other. All cell groups were then incubated for 30 minutes in the dark at 4°C. After two washing steps, the cells were treated with a Permeabilization/Fixation kit (Invitrogen (00-5523-00)). As the whole staining process was continued the next day, they were kept at 4°C in the dark overnight. The next day, the cells were treated with a Permeabilization buffer provided for the kit and then stained for Ki67 intracellularly. After incubating them for 30 minutes at 4°C in the dark and washing them, the cells were resuspended in FACS buffer containing counting beads (dilution 1:50) to measure the cell count. The stainings were then read out on a FACS Symphony^{TM} A5 Cell Analyzer. The steps described in this chapter (3.4.4. Staining protocol) were done by Camila Cancino. The results were then analysed with FlowJo and visualized in GraphPad Prism by me.

Marker	Fluorochrome	Dilution	Company	Clone
CD49d (=α4)	BV786	1:200	Biolegend	9F10
CCR4	BV750	1:50	BD	1G1
Integrin b7	BV711	1:800	Biolegend	FIB504
CD25	BV650	1:200	Biolegend	BC96
Ki67	BV551	1:800	Biolegend	Ki67
CCR6	BV605	1:100	Biolegend	G034E3
HLADR	BV570	1:200	Biolegend	L243
CD161	BV421	1:50	Biolegend	HP-3G10
LD	APC-efluor780	1:800	Thermofisher	
CD127	AlexaFluor 700	1:100	Biolegend	A019D5
GPR15	APC	1:100	Biolegend	SA302A10
CXCR3	PerCP-Cy5.5	1:100	BD	1C6/CXCR3
PD1	AlexaFluor 488	1:250	Biolegend	EH12.2H7
TIGIT	PE/Cy7	1:600	Biolegend	A15153G
CD29 (=β1)	PE/Cy5	1:1500	Biolegend	TS2/16
CCR9	PE/Dazzle 594	1:100	BD	11F2
CTLA4	PE	1:100	Biolegend	BNI3
CD103 (=αE)	BUV805	1:800	BD	Ber-ACT8
CCR7	BUV737	1:75	Thermofisher	3D12
CD45RA	BUV661	1:1500	BD	5H9
CD38	BUV615	1:400	BD	HIT2
CD8	BUV563	1:1500	BD	RPA-T8
CD3	BUV496	1:100	BD	UCHT1
CD4	BUV395	1:300	BD	RPA-T4

Table 6: Antibody panel

The antibodies, which go into both backbone and master mix are highlighted in black; the ones, which only go into the master mix are in red.

3.4.5 Review of comparability of samples

To make the handling of the samples easier, they were split up into twelve groups, which were then stained and analysed consecutively over several days. Each group also contained a sample of the same healthy control – a so-called "anchor control" -, to check that each group was handled the same way and could therefore be comparable. In Figure 7 a comparison of all anchor samples of the twelve groups is shown. It demonstrates that the groups are comparable, as the signal of different colours is similar. The staining of β1 did not work in one run. That is why its analysis sample size is smaller compared to the other flow cytometric data (see results).

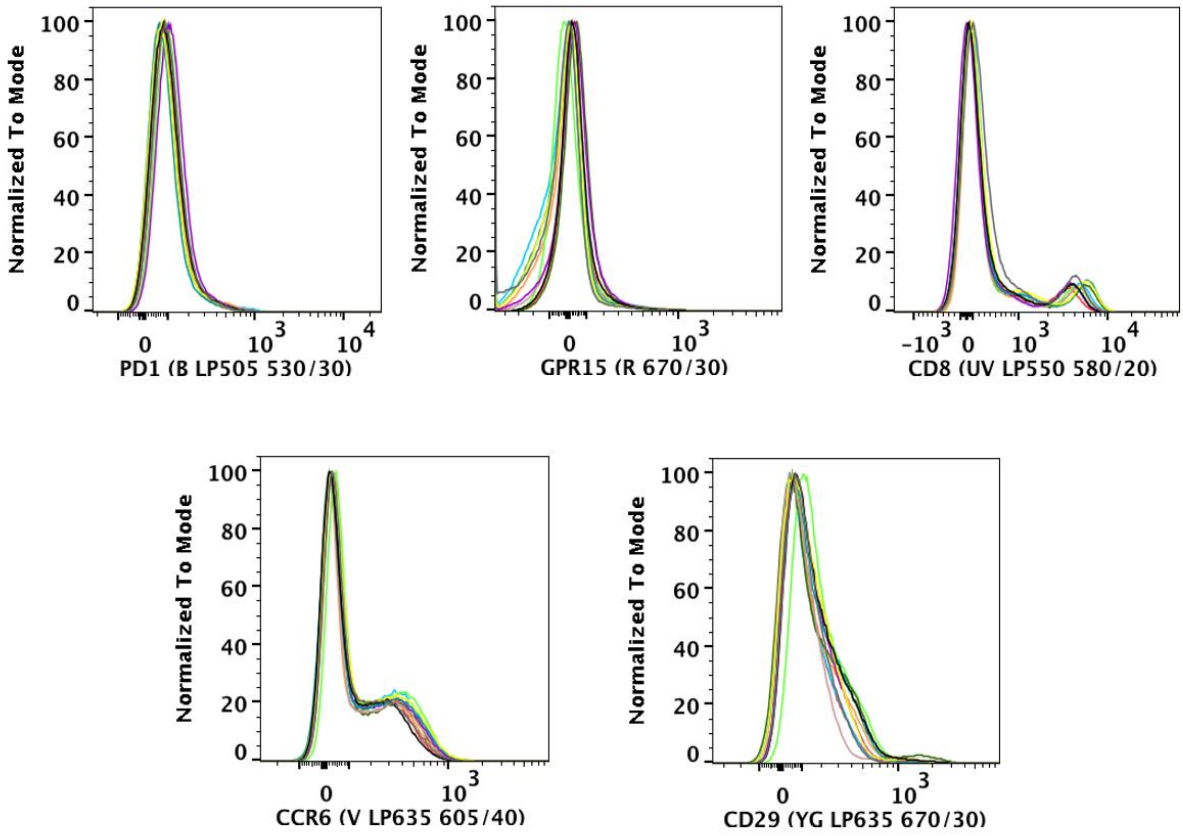


Figure 7: Comparison of anchor samples

Staining of one marker in all lasers was checked in all twelve groups of analysis runs

3.4.6 Data analysis

The FACS files, which were created by the FACS Diva software connected to the FACS Symphony, could be uploaded into the Flowjo program for further analysis. The gating strategy is shown in Figure 8.

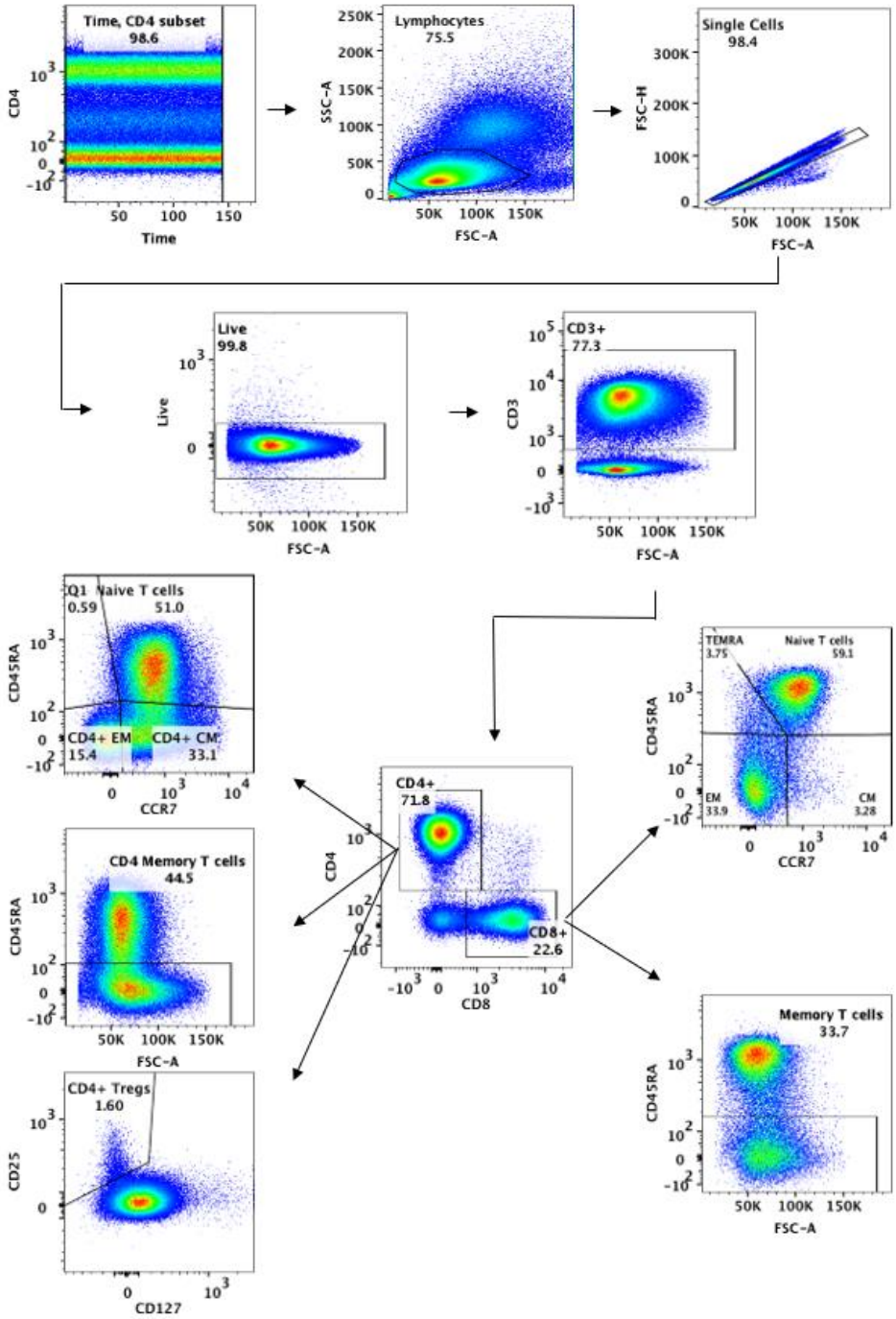


Figure 8: Gating strategy

After gating only on stable signals, the CD3$^+$ T lymphocytes were gated for by their granulation and width. Next, duplets and dead cells were filtered out. By gating on CD3$^+$ cells, the lymphocytes could be clearly analysed and differentiated into CD4$^+$- and CD8$^+$- T cells and their subgroups.

3.4.7 Statistical analysis

For the clinical markers, two different tests were used: To analyse differences in gender and treatment before using vedolizumab, Fisher´s exact test was used. For all other clinical markers – age, disease duration, leukocytes, lymphocytes, basophil, eosinophils, neutrophils, CrP, calprotectin, haemoglobin, MCV, MCH and thrombocytes – the Mann-Whitney-U-Test was used. The same test was used for analysing the trough level differences between responders and non-responders.

In the flow cytometry samples, the data of healthy donors and IBD patients before treatment were compared and analysed with the Mann-Whitney-U-Test, as well. Comparing T cell groups throughout the therapy at different time points, a Wilcoxon´s signed rank test was performed. Differences between responders and non-responders were statistically analysed with the Mann-Whitney-U-Test. The cell group affiliation of markers which were predictive for therapy response was analysed with a Wilcoxon´s signed rank test, as for example the percentage of memory T cells was also influenced by the percentage of naïve T cells. In the end, a Receiver operating characteristic (ROC) analysis was done for predictive markers to find the most optimal cut-off for therapy response.

3.4.8 Flow cytometry: Analysis approach

The Flow Cytometry data was analysed for four topics:

- Healthy donors were compared to all collected IBD patients before they started the therapy, to see how the disease influences the immune cell populations.
- The different time points of the initiation phase (week 0 = before therapy, week 2, week 6) were compared to each other to see how vedolizumab influences the cells
- Responders and non-responders were compared at the different time points of the initiation phase to identify potential markers which can predict early in the therapy or even before, whether the patient will respond to the therapy. Consequently, only the first three time points of the therapy – the initiation phase – were analysed with Flow cytometry: Only a marker which can be detected early can help in the clinical decision-making.
- If a significant difference was found in the percentage numbers, the absolute cell numbers were calculated to validate, whether the percentage shift was caused by a change in the examined subpopulation itself or due to a large in- or decrease of another subpopulation, which therefore would shift the relative numbers of all subpopulations.

4. Results

4.1 Patients' characteristics at baseline

In the time range between July 2018 and November 2021, 52 patients were induced with vedolizumab and included into the study. Two patients had to be excluded from the analysis: One did not continue the study after week 2 and the other did not bear enough clinical scoring data to determine his response status. From the 50 remaining patients 24 could be classified as responders and 14 as non-responders. 12 patients were classified as remitters as they continued the therapy but failed to show clear clinical differences, which would have underlined their therapy response or non-response. It was rather proposed that the inflammation level in their gut was low from the beginning of the therapy and continued to be so.

All the basic information about the patient cohort is shown in Table 7. Patients with CD, UC and indeterminate colitis – not clearly differentiated patients between UC and CD - were included. Around three-quarters of patients had UC. All three groups are well balanced in the age range with an average age ranging from 41 to 52 years old. Gender is also balanced in all groups except for CD, which the low number of patients can explain. Patients had on average a mild to moderate disease at the beginning of the therapy. The low average rate can be attributed to the inclusion of patients in remission. The disease localisation was balanced in CD patients, whereas UC patients mostly had an inflammation in the left side of the colon or an extensive inflammation in the entire colon. More than half of the cohort received no comedication but a few received more than one.

A control group of 26 healthy controls, which were age and gender balanced, was collected as well.

		Control	Crohn's disease	Ulcerative colitis	Indeterminate colitis
Number of patients		26	9	39	2
Age (Ø, range)		41 (22-75)	45 (23-75)	42 (19-77)	52 (51-53)
Female (%)		42.31	88.9	51.28	50
HBI (Ø, range)			9.44 (4-27)		
Partial Mayo Score (Ø, range)				2.91 (0-7)	
Comedication (%)	None		55.56	51.28	100
	Prednisolone		22.22	35.9	
	Azathioprine		22.22	2.56	
	Budesonide			2.56	
	Prednisolone + Cyclosporine			5.13	
	Infliximab			2.56	
Disease localisation (%)			L1 (ileal): 11.11	E1 (proctitis): 15.38	
			L2 (ileocolonic): 11.11	E2 (left-sided): 43.59	
			L3 (colonic): 22.22	E3 (extensive): 38.46	
			L1/L2 (ileocolonic): 22.22	Not defined: 2.56	
			L1/L4: 11.11		
			L3/4 (colonic): 11.11		
			Not defined: 11.11		

Table 7: Patients' characteristics at baseline (all patients)

Not all patients were included in all experiments. This can be mainly explained as for the comparison of clinical markers and trough levels only responders and non-responders were used, whereas remitters were excluded. How many patients were included in which experiments and which are used in multiple experiments is shown in Figure 9.

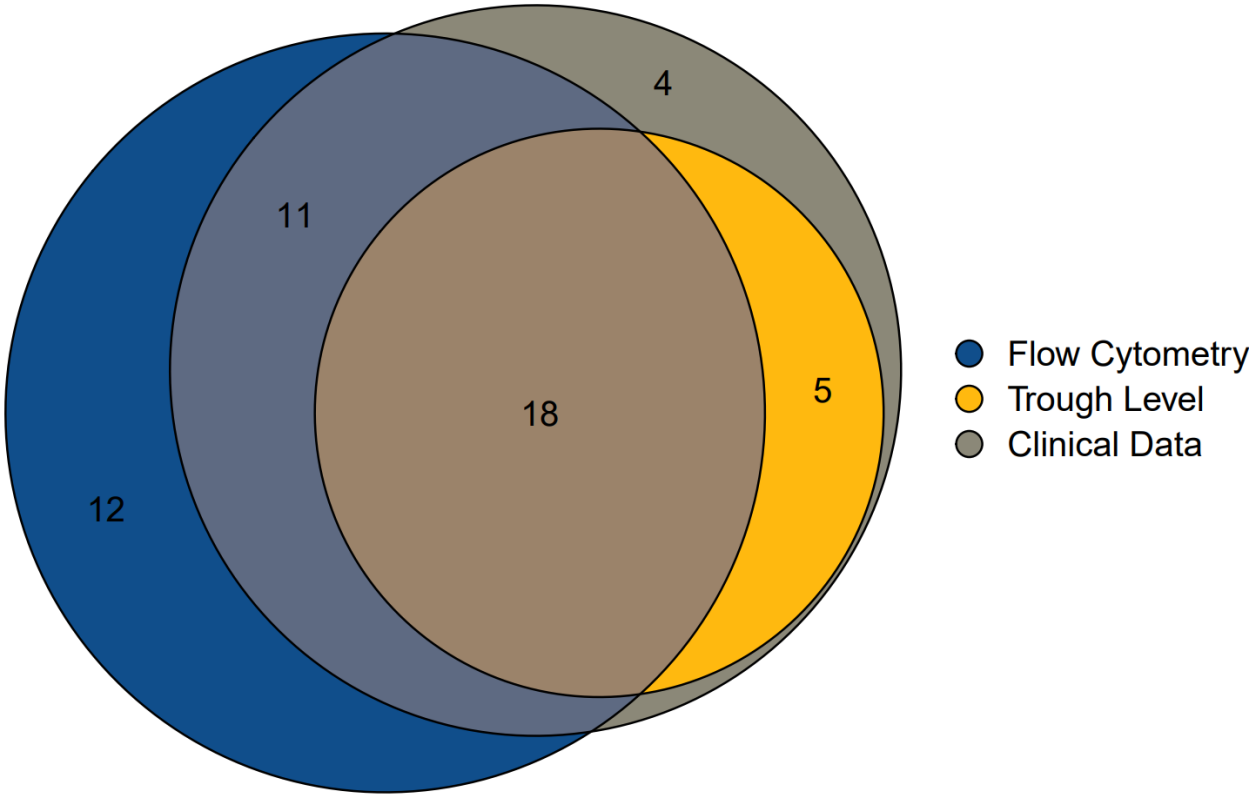


Figure 9: Overview of usage of patient data

Flow cytometric data could be analysed from 41 patients in total, followed by the clinical data with 38 patients. Through level could only be measured in 23 patients. 18 patients were included in all three analysis branches.

The characteristics of the 38 patients used for the clinical analysis, are shown in Table 8: Fewer patients were included in this analysis, as only responders and non-responders are included. That is why there are fewer UC patients and no indeterminate colitis patients are included. Looking at the changed composition of the UC patients age, gender and disease localisation are quite similar in percentage numbers compared to the whole cohort. The patients only received less comedication and had a higher Partial Mayo Score at the beginning of therapy.

		Crohn's disease	Ulcerative colitis
Number of patients		9	29
Age (Ø, range)		45 (23-75)	42 (19-77)
Female (%)		88.89	55.17
HBI (Ø, range)		9.44 (4-27)	
Partial Mayo Score (Ø, range)			4 (0-7)
Comedication (%)	None	55.56	37.93
	Prednisolone	22.22	44.83
	Azathioprine	22.22	3.45
	Infliximab		3.45
	Prednisolone + Cyclosporine		6.9
	Budesonide		3,45
Disease localisation (%)		L1 (ileal): 22.22	E1 (proctitis): 24.14
		L2 (ileocolonic): 11.11	E2 (left-sided): 41.38
		L3 (colonic): 22.22	E3 (extensive): 34.48
		L1/L2 (ileocolonic): 22.22	
		L1/L4: 11.11	
		L3/4 (colonic): 11.11	

Table 8: Patients' characteristics at baseline (clinical data)

For the trough level analysis only responders and non-responders were used (see Table 9, collected at week 6). However, as the collection of serum samples was started at a later time point after the study initiation hence fewer patients could be included for this analysis. For both UC and CD, the median age level is similar to the whole cohort, whereas fewer females are in the trough level group. They also received less comedication. Furthermore, the collected samples showed less inflammation: This can be explained as the samples were collected at week 6 – at the end of the induction phase of the treatment. The disease localisation for CD is quite comparable, whereas most UC patients have a proctitis in the trough level cohort.

		Control	Crohn's disease	Ulcerative colitis
Number of patients		0	6	17
Age (Ø, range)			44 (23-65)	40 (20-77)
Female (%)			83.33	41.18
HBI (Ø, range)			5.5 (0-24)	
Partial Mayo Score (Ø, range)				1.93 (0-7)
Comedication (%)	None		50	41.18
	Prednisolone		16.67	41.18
	Azathioprine		33.33	5.88
	Infliximab			5.88
	Prednisolone + Cyclosporine			5.88
Disease localisation (%)			L2 (ileocolonic): 16.67	E1 (proctitis): 29.42
			L3 (colonic): 33.33	E2 (left-sided): 41.18
			L1/L2 (ileocolonic): 16.67	E3 (extensive): 29.42
			L1/L4: 16.67	
			L3/4 (colonic): 16.67	

Table 9: Patients' characteristics at week 6 (trough level)

Looking at the group used in Flow cytometry (see Table 10) all CD patients and indeterminate colitis´ patients of the whole cohort could be included. The fewer UC patients, which were collected, were mostly female. However, comedication, disease localisation and age are comparable to the whole group (see Table 10).

		Control	Crohn's disease	Ulcerative colitis	Indeterminate colitis
Number of patients		26	9	30	2
Age (Ø, range)		41.04 (22-75)	45 (23-75)	40 (19-74)	52 (51-53)
Female (%)		42.31	88.9	60	50
HBI (Ø, range)			9.44 (4-27)		
Partial Mayo Score (Ø, range)				2.93 (0-7)	
Comedication (%)	None		55.56	56.67	100
	Prednisolone		22.22	33.33	
	Azathioprine		22.22	3.33	
	Budesonide			3.33	
	Prednisolone + Cyclosporine			3.33	
			L1 (ileal): 22.22		
Disease localisation (%)			L2 (ileocolonic): 11.11	E1 (proctitis): 13.33	
			L3 (colonic): 22.22	E2 (left-sided): 50	
			L1/L2 (ileocolonic): 22.22	E3 (extensive): 36.67	
			L1/L4: 11.11		
			L3/4 (colonic): 11.11		

Table 10: Patients' characteristics at baseline (flow cytometry)

4.2 Standard clinical markers

4.2.1 Basic patient information

I first analysed the basic markers, such as age and gender: Both did not show significant predictive power for therapy response to vedolizumab. Responders were on average younger (40 vs. 48.07, see Figure 10a) and more male patients turned out to be responders to vedolizumab treatment (male: 64.29% responders vs. 35.71% non-responders; female 62.5% responders vs. 37.5% non-

responders, see Figure 10b). However, both markers were not statistically significantly predictive of the therapy outcome.

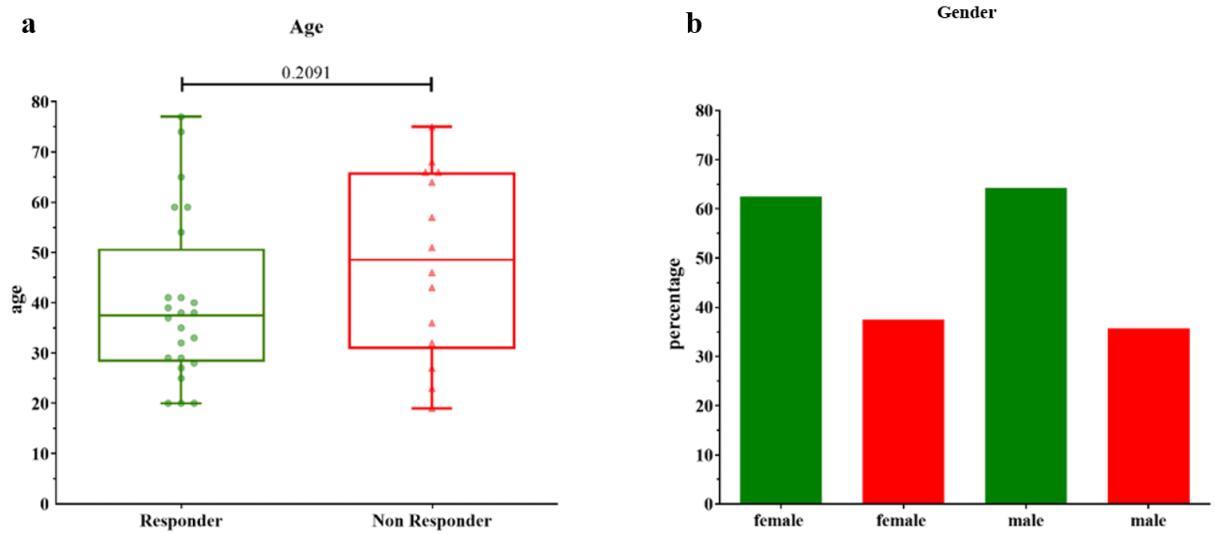


Figure 10: Comparison of age and gender between responders and non-responders

***a**: Comparison of the age between responders and non-responders; n (responders) = 24; n (non-responders) = 14; test used: Mann-Whitney Test*
***b**: Comparison of the gender between responders and non-responders; n (responders) = 24; n (non-responders) = 14; test used: Fisher´s exact test; p = 1*

4.2.2 Clinical history

Furthermore, the time since the diagnosis of the patients' disease and treatment with any anti-TNF-drugs before using vedolizumab were analysed regarding therapy response. Non-Responders had on average a longer disease duration (11.45 vs. 8.75 years, see Figure 11a). But the difference was not significant enough to predict response. The previous anti-TNF-treatment was also not predictive for therapy response to vedolizumab with 65.22% of anti-TNF treated and 60% of anti-TNF naïve being responders (see Figure 11b).

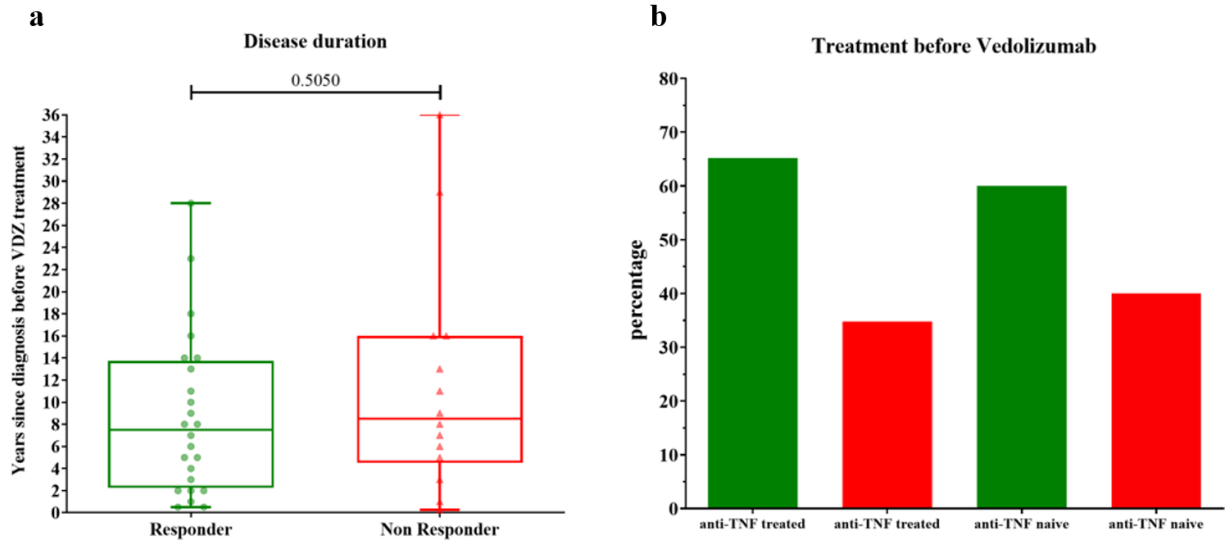


Figure 11: Comparison of disease duration and treatment before vedolizumab between responders and non-responders

***a**: Comparison of the disease duration (time from diagnosis until vedolizumab was used) between responders and non-responders; n (responders) = 24; n (non-responders) = 14; test used: Mann-Whitney Test*
***b**: Comparison of the treatment before vedolizumab between responders and non-responders; green = responders, red = non-responders; n (anti-TNF treated) = 23; n (anti-TNF naive) = 15; test used: Fisher´s exact test; p = 0,7462*

4.2.3 Inflammatory Markers

Medical doctors regularly use clinical inflammatory markers such as CrP, leukocytes and calprotectin to assess the inflammation status in a patient. They are also being assessed in patients with IBD to evaluate the change and status of the disease.

A reduction in leukocyte count can be monitored in both responders and non-responders over time (see Figure 12). On average the leukocyte count is higher in responders at the beginning of the therapy (9.55 vs 8.72/nl) and with the beginning of the therapy drops below the level of non-responders (week 2: 7.77 vs. 8.12; week 6: 7.84 vs. 8.83; week 18: 6.72 vs. 7.27). Nevertheless, these comparisons stay insignificant (test used: Mann-Whitney-Test; p (w0) = 0.535; p (w2) = 0.5526; p (w6) = 0.2539; p (w18) = 0.5617).

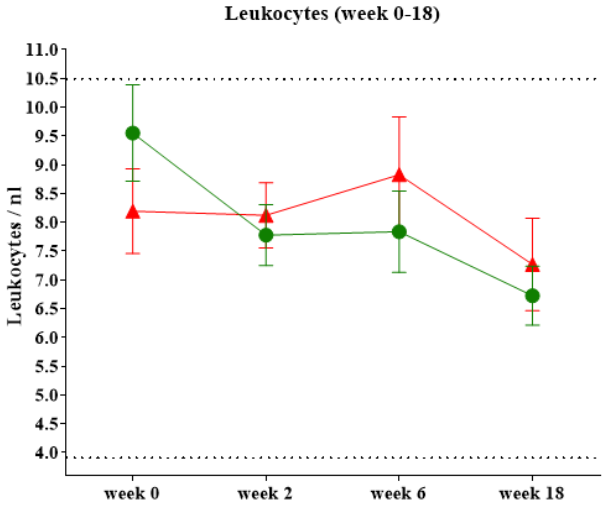


Figure 12: Comparison of leukocyte level between responders and non-responders

shown: standard error of mean; green = responders; red = non-responders; dotted lines represent the lower and upper limit of the average healthy population;
responders: n (week 0) = 24; n (week 2) = 10; n (week 6) = 20; n (week 18): 17
non-responders: n (week 0) = 13; n (week 2) = 7; n (week 6) = 9; n (week 18): 6
test used: Mann-Whitney test; p (week 0) = 0.535; p (week 2) = 0.5526; p (week 6) = 0.2539; p (week 18) = 0.5617

The leukocyte subsets lymphocytes, monocytes, basophils, eosinophils and neutrophils were also analysed for their predictive power for therapy response to vedolizumab. However, there were no significant differences found at any time point between responders and non-responders (see Figure 13).

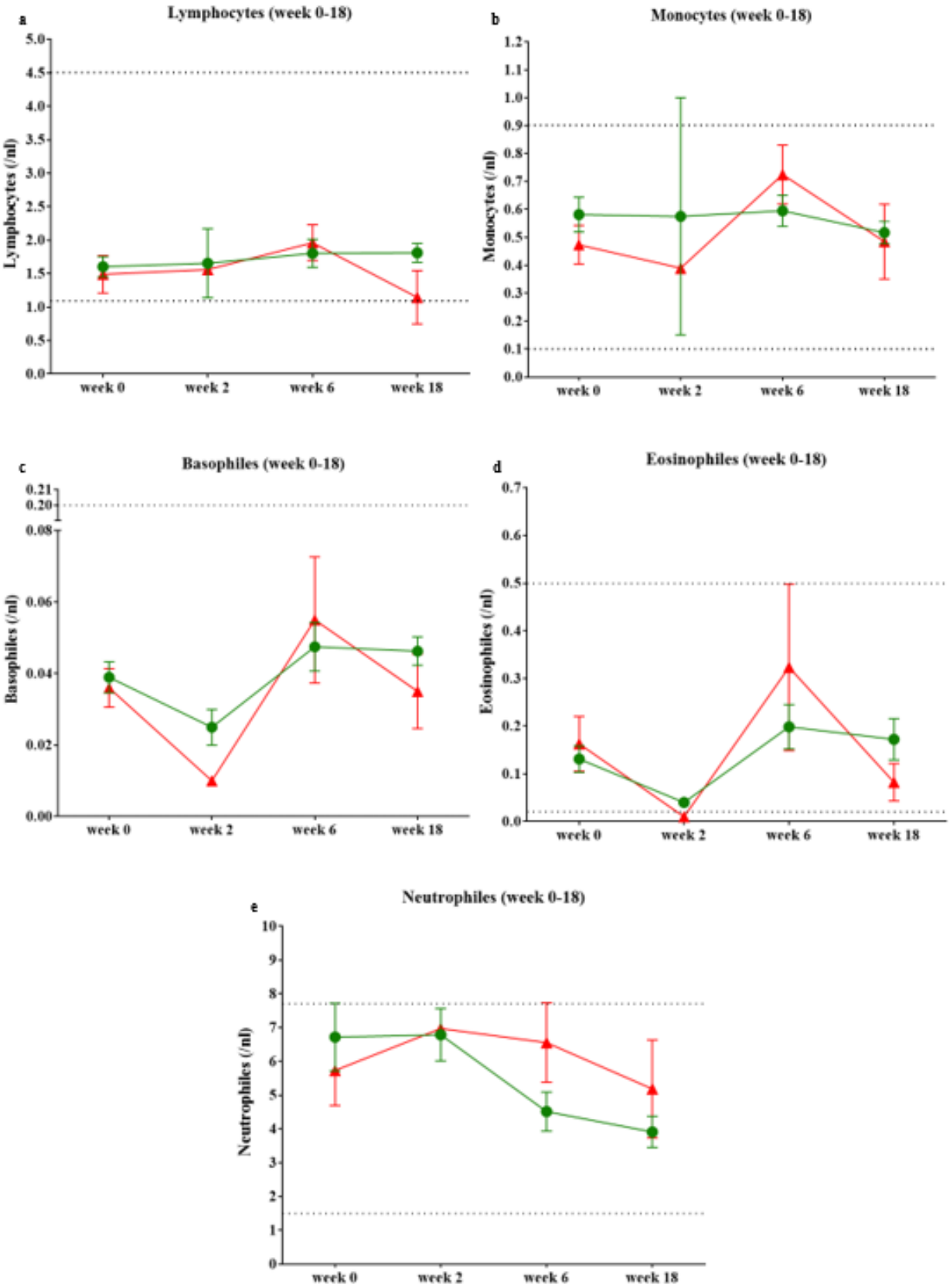

a
Lymphocytes (week 0-18)
Lymphocytes (/nl)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
week 0
week 2
week 6
week 18
b
Monocytes (week 0-18)
Monocytes (/nl)
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
week 0
week 2
week 6
week 18
c
Basophiles (week 0-18)
Basophiles (/nl)
0.21
0.20
0.08
0.06
0.04
0.02
0.00
week 0
week 2
week 6
week 18
d
Eosinophiles (week 0-18)
Eosinophiles (/nl)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
week 0
week 2
week 6
week 18
e
Neutrophiles (week 0-18)
Neutrophiles (/nl)
10
9
8
7
6
5
4
3
2
1
0
week 0
week 2
week 6
week 18

Figure 13: Comparison of leukocyte subgroups between responders and non-responders

in all figures: shown: standard error of mean; green = responders; red = non-responders; dotted lines represent the lower and upper limit of the average healthy population
in all figures: responders: n (week 0) = 20; n (week 2) = 2; n (week 6) = 8; n (week 18): 16; non-responders: n (week 0) = 10; n (week 2) = 1; n (week 6) = 6; n (week 18): 4; test used: Mann-Whitney test
***a**: Comparison of the lymphocyte count between responders and non-responders; p (week 0) = 0.5656; week 2 not enough datapoints; p (week 6) = 0.4908; p (week 18) = 0.2054*
***b**: Comparison of the monocyte count between responders and non-responders; p (week 0) = 0.3117; week 2 not enough datapoints; p (week 6) = 0.7829; p (week 18) = 0.8076*
***c**: Comparison of the basophile count between responders and non-responders; p (week 0) = 0.751; week 2 not enough datapoints; p (week 6) = 0.9094; p (week 18) = 0.3125*
***d**: Comparison of the eosinophil count between responders and non-responders; p (week 0) = 0.8477; week 2 not enough datapoints; p (week 6) = 0.9755; p (week 18) = 0.2576*
***e**: Comparison of the neutrophil count between responders and non-responders; p (week 0) = 0.6496; week 2 not enough datapoints; p (week 6) = 0.1812; p (w18) = 0.4372*

The results are more mixed for CrP (see Figure 14): At week 0, CrP in responders is on average lower than in non-responders (7.17 vs. 11.39 mg/l). At week 2, the mean value increases swiftly to 16.16 mg/l above the average value of non-responders (10.45 mg/l). But at week 6 and 18 CrP is on average lower in responders than non-responders (week 6: 3.24 vs. 20.625 mg/l; week 18: 3.21 vs. 14.27 mg/l). With these numbers, responders also go below the reference value level of 5mg/l. The comparison of CrP between responders and non-responders is however insignificant, except for week 6 (test used: Mann-Whitney-Test; p (w0) = 0.2796; p (w2) = 0.2564; p (w6) = 0.0191; p (w18) = 0.0863).

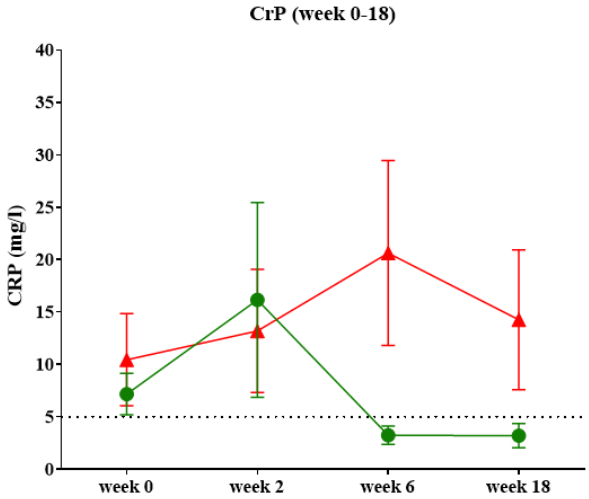


Figure 14: Comparison of CrP between responders and non-responders

shown: standard error of mean; green = responders; red = non-responders; dotted line represents the upper limit of the average healthy population;
responders: n (week 0) = 24; n (week 2) = 10; n (week 6) = 19; n (week 18): 16
non-responders: n (week 0) = 13; n (week 2) = 6; n (week 6) = 8; n (week 18): 6
test used: Mann-Whitney test; p (week 0) = 0.2796; p (week 2) = 0.2564; p (week 6) = 0.0191; p (week 18) = 0.0863

Calprotectin shows a better picture than the other markers (see Figure 15): At week 0 both groups start at close average levels of 387.35 µg/g for responders and 394.38 µg/g for non-responders. This stays similar for week 2 (responders 469 µg/g; non-responders: 438 µg/g). But with week 6 the two groups can be clearly separate: The value for responders decreases rapidly, whereas the calprotectin level rises quickly in non-responders. That is why the calprotectin level at week 6 and 18 is statistically significantly different between responders and non-responders (test used: Mann-Whitney-Test; p (w6) = 0.0241; p (w18) = 0.0012).

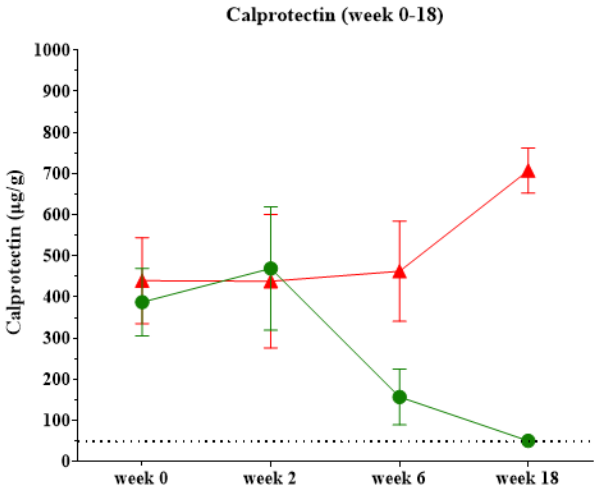


Figure 15: Comparison of calprotectin level between responders and non-responders

shown: standard error of mean; green = responders; red = non-responders; dotted line represents the upper limit of the average healthy population;
responders: n (week 0) = 18; n (week 2) = 5; n (week 6) = 12; n (week 18): 6
non-responders: n (week 0) = 10; n (week 2) = 6; n (week 6) = 7; n (week 18): 7
test used: Mann-Whitney test; p (week 0) = 0.5807; p (week 2) = 0.9351; p (week 6) = 0.0241; p (week 18) = 0.0012

4.2.4 Erythrocytic Markers

The inflammation in the gut of IBD patients causes the reduction of a lot of important nutrients and micronutrients such as iron and Vitamin B12. The two have an important influence on the production of erythrocytes. Therefore, a reduction in intestinal transportation of iron and Vitamin B12 causes a decrease in several haematological markers, such as erythrocyte count, haemoglobin, mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH).

Erythrocyte count and haemoglobin show similar curves (see Figure 16). The baseline in responders and non-responders is equal at week 0 for both markers with an erythrocyte count of 4.57/pl in responders and 4.53/pl in non-responders on average. The haemoglobin level is at 13.25 g/dl in responders and 13.24 g/dl in non-responders. Over week 2 and 6, the erythrocyte count and haemoglobin level rise in responders and drop in non-responders so much that at week 6 the difference in the haemoglobin level is significant (test used: Mann-Whitney-Test; p (w6) = 0.0364). At week 18 the remaining non-responders levels rise again to surpass even the levels of the responders: Responders have an erythrocyte count of 4.64/pl and non-responders of 4.78/pl on average. The haemoglobin level is at 13.53 g/dl in responders and 14.24 in non-responders. The differences stay insignificant.

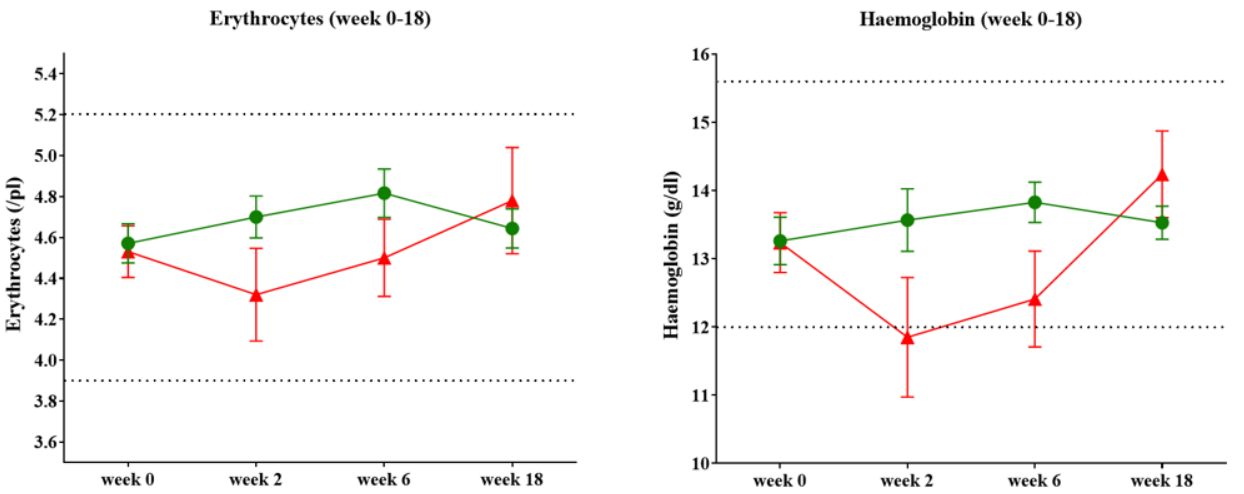


Figure 16: Comparison of erythrocyte count and haemoglobin level between responders and non-responders

in both figures: shown: standard error of mean; green = responders; red = non-responders; dotted lines represent the lower and upper limit of the average healthy population;
in both figures: responders: n (week 0) = 24; n (week 2) = 9; n (week 6) = 18; n (week 18): 17; non-responders: n (week 0) = 13; n (week 2) = 6; n (week 6) = 9; n (week 18): 5; test used: Mann-Whitney test
***a**: Comparison of erythrocyte count between responders and non-responders; p (week 0) = 0.8562; p (week 2) = 0.1633; p (week 6) = 0.1966; p (week 18) = 0.8877*
***b**: Comparison of haemoglobin level between responders and non-responders; p (week 0) = 0.9062; p (week 2) = 0.1534; p (week 6) = 0.0364; p (week 18) = 0.1577*

The trend of mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH) also tend to follow similar pathways (see Figure 17). The starting points at week 0 in responders and non-responders are very close again with MCV being at 87.19fl in responders and 86.64fl in non-responders and MCH at 29.04pg in responders and 29.2pg in non-responders on average. Over week 2 and 6, the levels drop in non-responders. This can be especially seen at week 6, at which the difference in MCV and MCH turns significant between the two groups (test used: Mann-Whitney-Test; MCV: p (w6): 0.0185; MCH: p (w6) = 0.0364). These low levels are a sign of an anaemia of a chronic disease. The few remaining non-responders have then a rise in their MCV and MCH, as in haemoglobin level and erythrocyte count: The MCV lies at 85.95fl in responders and at 87.4fl in non-responders, whereas MCH is at 29.18pg in responding patients and 30.04pg in non-responding patients to vedolizumab. These differences stay insignificant (test used: Mann-Whitney-Test; MCV: p(w18) = 0.9866; MCH: p(w18) = 0.8947).

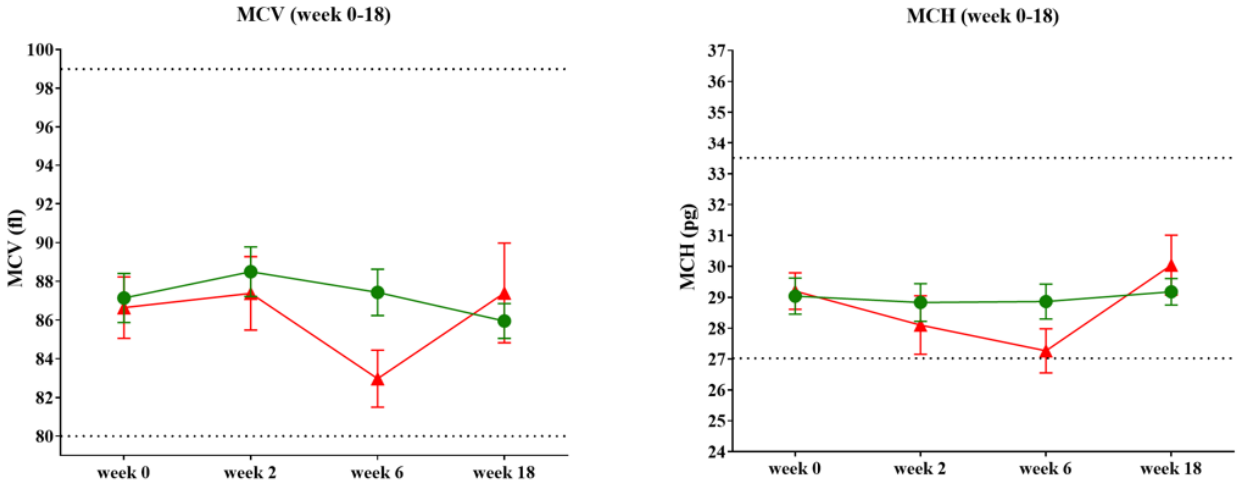


Figure 17: Comparison of MCV and MCH between responders and non-responders

in both figures: shown: standard error of mean; green = responders; red = non-responders; dotted lines represent the lower and upper limit of the average healthy population;
in both figures: responders: n (week 0) = 24; n (week 2) = 9; n (week 6) = 18; n (week 18): 17; non-responders: n (week 0) = 13; n (week 2) = 6; n (week 6) = 9; n (week 18): 5; test used: Mann-Whitney test
***a**: Comparison of MCV between responders and non-responders; p (week 0) = 0.429; p (week 2) = 0.6496; p (week 6) = 0.0185; p (week 18) = 0.9866*
***b**: Comparison of MCH between responders and non-responders; p (week 0) = 0.7715; p (week 2) = 0.4545; p (week 6) = 0.0364; p (week 18) = 0.8947*

4.2.5 Thrombocyte level

The thrombocyte level was compared in responders and non-responders (see Figure 18). While beginning at similar levels at week 0 with 310.58/nl in responders and 317.92/nl, the thrombocyte number rises in non-responders and drops in responders over week 2 and 6 to the point that there is a significant difference at week 6 (test used: Mann-Whitney-Test; p (w6) = 0.0239). At week 18, the number steeply drops in non-responders to 210.2/nl with responders at 278.59/nl. This comparison stays insignificant (test used: Mann-Whitney-Test; p (w18) = 0.2488).

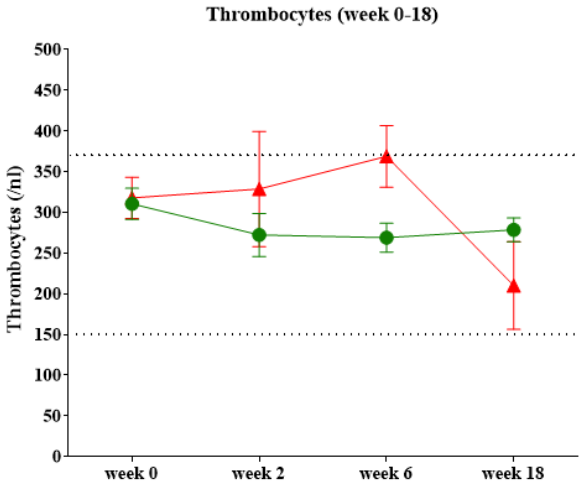


Figure 18: Comparison of thrombocyte level between responders and non-responders

shown: standard error of mean; green = responders; red = non-responders; dotted line represents the upper limit of the average healthy population;
responders: n (week 0) = 24, n (week 2) = 9, n (week 6) = 17, n (week 18): 17
non-responders: n (week 0) = 13, n (week 2) = 5, n (week 6) = 9, n (week 18): 5
test used: Mann-Whitney test; p (week 0) = 0.632, p (week 2) = 0.4376, p (week 6) = 0.0239, p (week 18) = 0.2488

4.2.6 Vedolizumab trough level

Like many other studies, I also compared vedolizumab trough levels at week 6 between responders and non-responder. I could not show a significant difference between the two groups: Even though the average trough level is higher in responders than in non-responders (30.73 µg/ml vs. 21.88 µg/ml), no significance can be shown with p = 0.3572 (see Figure 19).

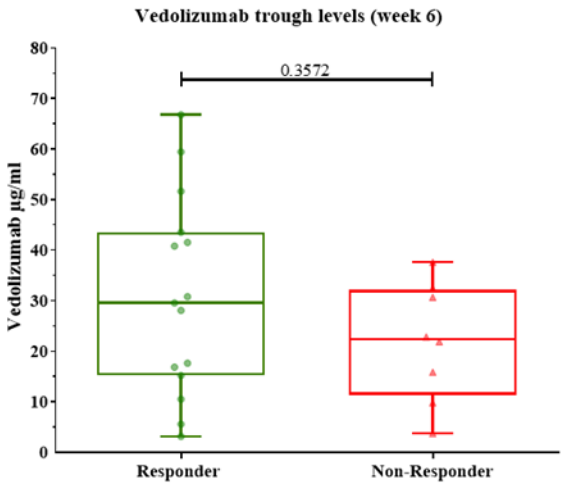


Figure 19: Comparison of vedolizumab trough level between responders and non-responders

shown: standard deviation; n (responders) = 15; n (non-responders) = 8; test used: Mann-Whitney test

4.3 Assessment of T cells before and after vedolizumab induction

4.3.1 Measurement of α4β7-Integrin with bound vedolizumab

Before analysing the isolated peripheral blood mononuclear cells (PBMCs) the question had to be answered whether α4β7-Integrin-expression could still be measured, as vedolizumab binds to the integrin-complex. A possibility could be that vedolizumab would bind to all epitopes of α4β7-Integrin and thereby make it impossible to measure its expression. Therefore, the PBMCs of three healthy controls were treated with vedolizumab in different concentrations. Afterwards, they were stained with an antibody mix to gate on $CD4^+$ memory T cells (see Figure 20a) and analyse their α4β7-Integrin expression. In Figure 20b the flow cytometry plots of one patient show that the experiment worked, as with a rising concentration of unmarked vedolizumab, the signal of marked vedolizumab, which was added afterwards, decreases. The flow cytometry plots in Figure 20c show that α4β7-Integrin-expression can still be measured and does not get affected by any concentration of vedolizumab with which it is blocked. Figure 21 shows the underlying data of all three patients to proof it.

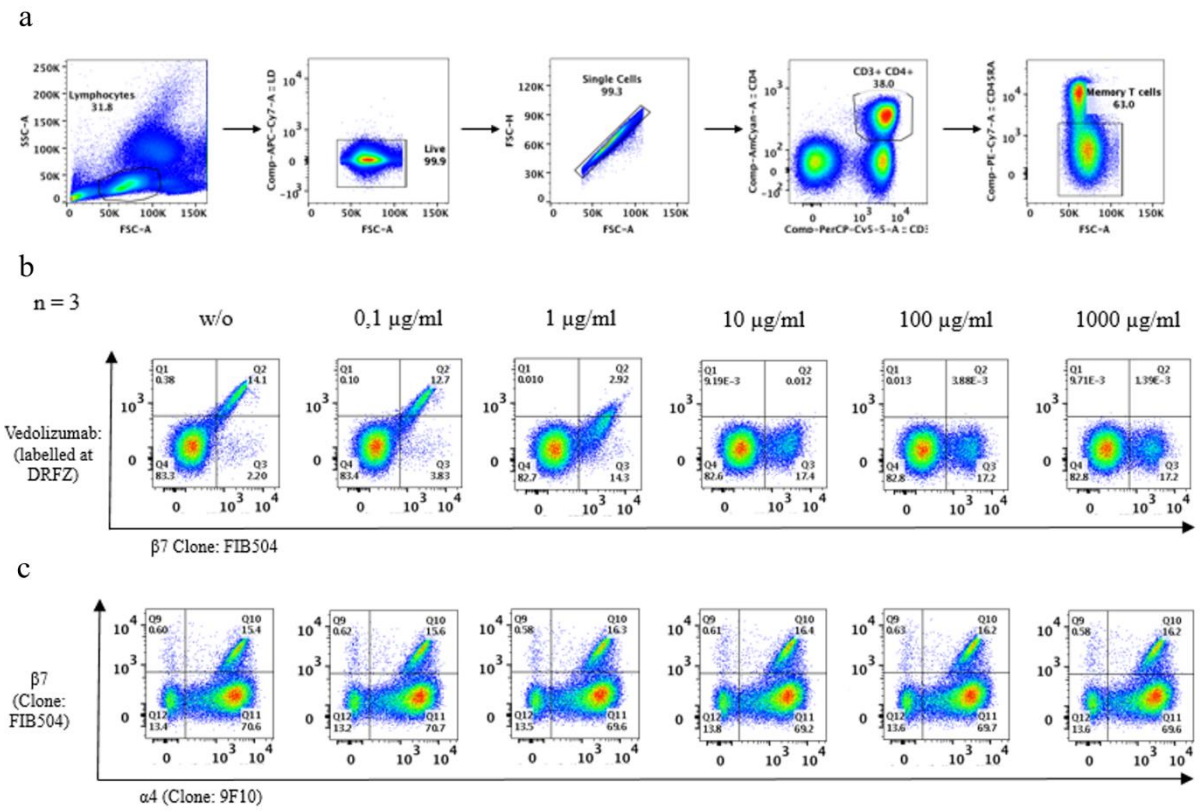


Figure 20: Measurement of $\alpha4^+\beta7^+$ while blocking with vedolizumab

a: Gating strategy
b: Measuring vedolizumab and β7 expression on $CD4^+$ Memory T cells
c: Measuring α4 and β7 expression on $CD4^+$ Memory T cells

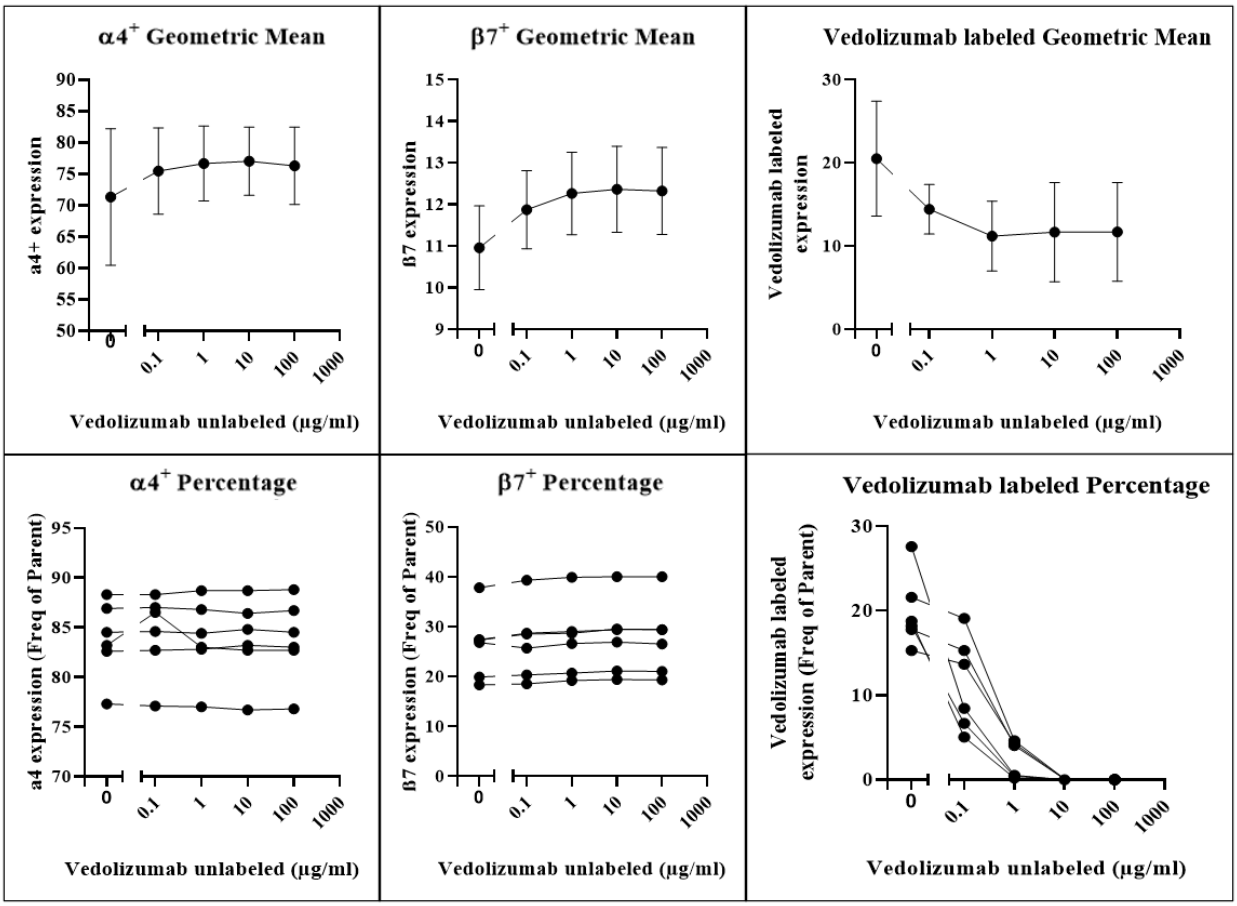


Figure 21: Measurement of Geometric Mean and Percentage of α4+, β7+ and labelled vedolizumab with unmarked vedolizumab

(n = 6 healthy donors)

4.3.2 CD3+: T lymphocytes

After gating on single cells, which were alive, $CD3^+$ T cells were analysed: Significantly lower levels in both relative and absolute numbers could be shown in IBD patients before therapy in comparison to healthy donors (median: 68.1% vs. 74.85% and absolute numbers: 2022 vs. 2445). The percentage numbers, not the absolute ones, then rise statistically significantly in week 2 to 69.4%. This trend does not statistically significantly continue in week 6 as there is no significant difference between week 0 and week 6 in relative numbers (see Figure 22).

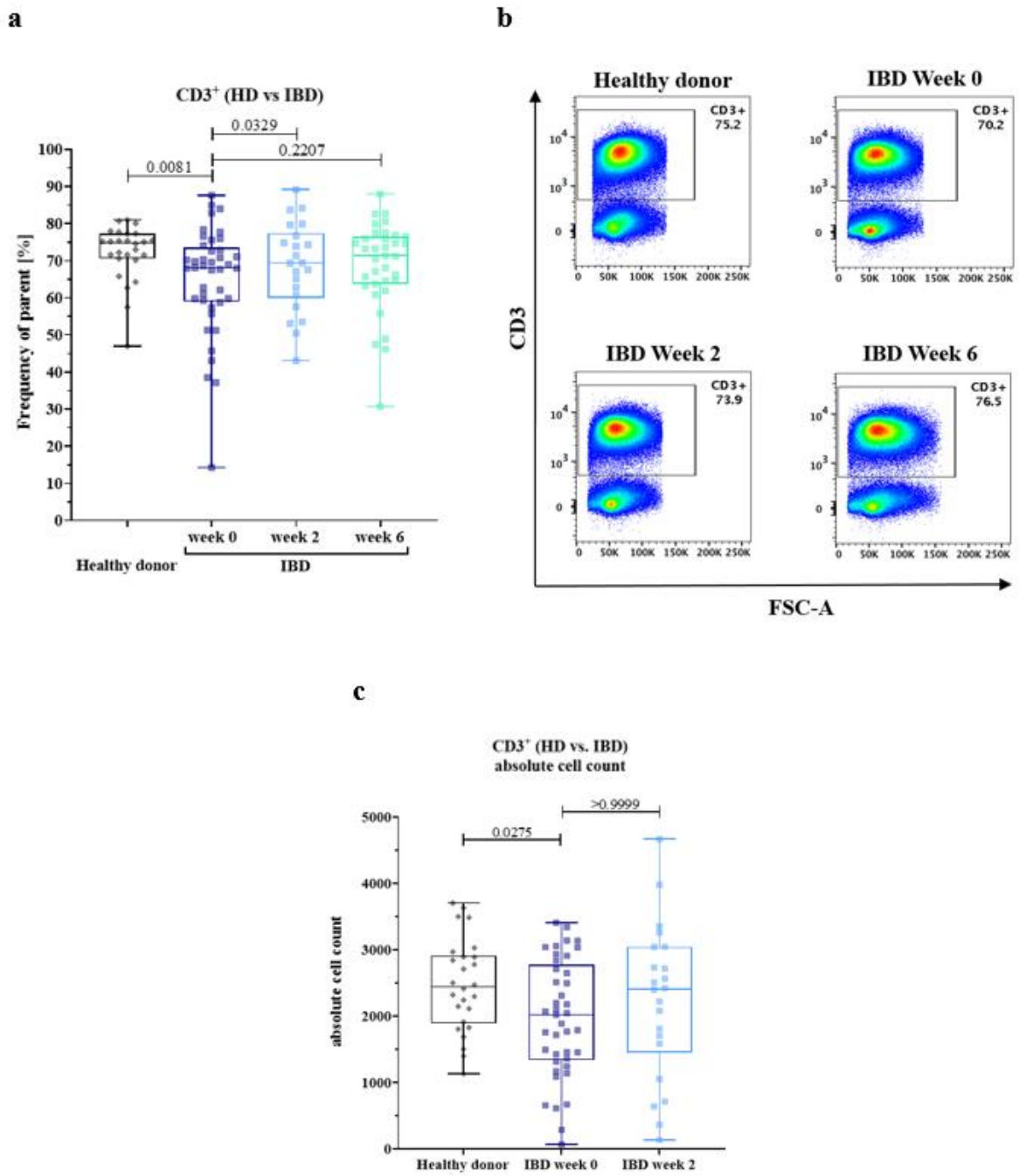


Figure 22: CD3+ T cells: Comparison of healthy donors and IBD patients

(gated on live cells-> single cells)
***a**: Comparison of healthy donors and IBD patients (frequency of parent); n (HD) = 26; IBD: n (week 0) = 41; n (week 2) = 22; n (week 6) = 35; test used for HD vs IBD week 0: Mann-Whitney Test; test used for IBD week 0 vs week 2 and week 6: Wilcoxon Test*
***b**: Examples of flow cytometry plots showing the differences between healthy donors and the different time points*
***c**: Comparison of healthy donors and IBD patients at week 0 and 2 (absolute cell count); same cohort and tests used as in a*

The CD3$^+$ T lymphocyte numbers of responders are higher on median than non-responders in week 0 and week 2 (week 0: 68% vs. 63.65%; week 2: 65.1% vs. 62.8%). Then, this ratio turns around in week 6 (64.6% vs. 70.15%). However, no statistically significant difference could be found at any of the three analysed time points between responders and non-responders (see Figure 23).

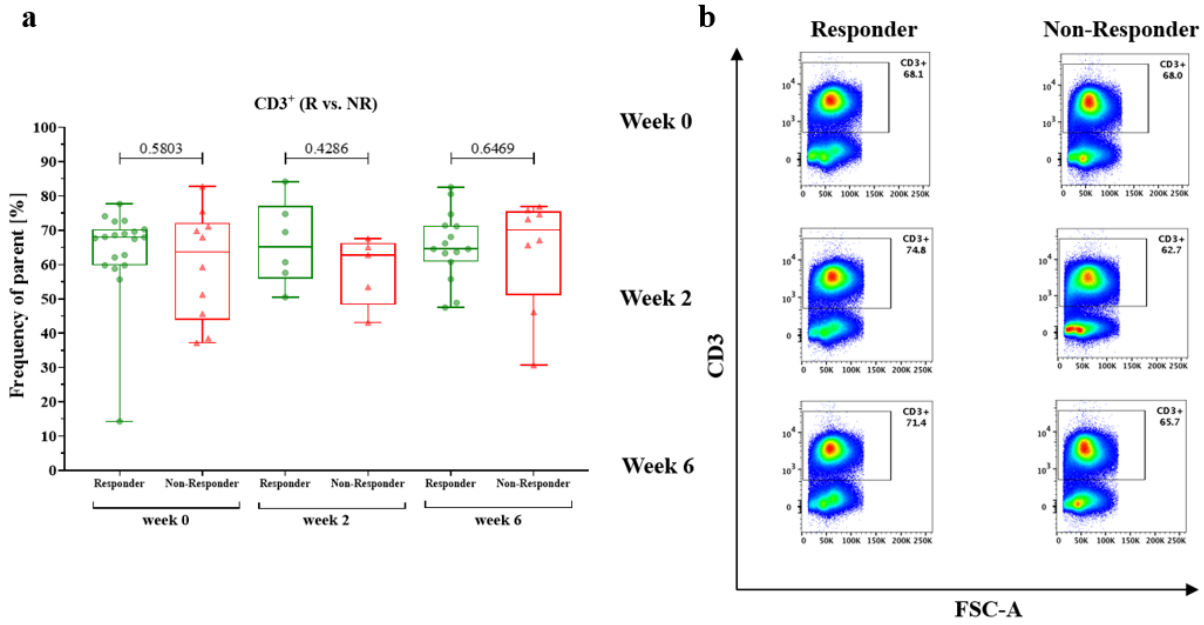


Figure 23: CD3$^+$ T cells: Comparison of responders and non-responders

(gated on live cells -> single cells -> CD3$^+$)
***a**: Comparison of responders and non-responders; responder: n (week 0) = 19; n (week 2) = 6; n (week 6) = 15; non-responder: n (week 0) = 10; n (week 2) = 5; n (week 6) = 8; test used: Mann-Whitney-Test*
***b**: Examples of flow cytometry plots showing the differences between responders and non-responders during the different weeks*

4.3.3 CD4$^+$ and CD8$^+$ T lymphocytes

After checking all T lymphocytes, the subsets CD4$^+$ and CD8$^+$ T lymphocytes were analysed. Comparing healthy donors to IBD patients before treatment, no significant difference could be detected in either CD4$^+$ or CD8$^+$ T cells: The percentage numbers of CD4$^+$ T cells in IBD patients at week 0 are slightly lower on median than in healthy donors (63% vs. 65.1%; see Figure 24b), whereas the CD8$^+$ portion at week 0 is higher in IBD patients than in healthy donors (30% vs. 25.6%; see Figure 24c). During therapy no major shifts could be detected.

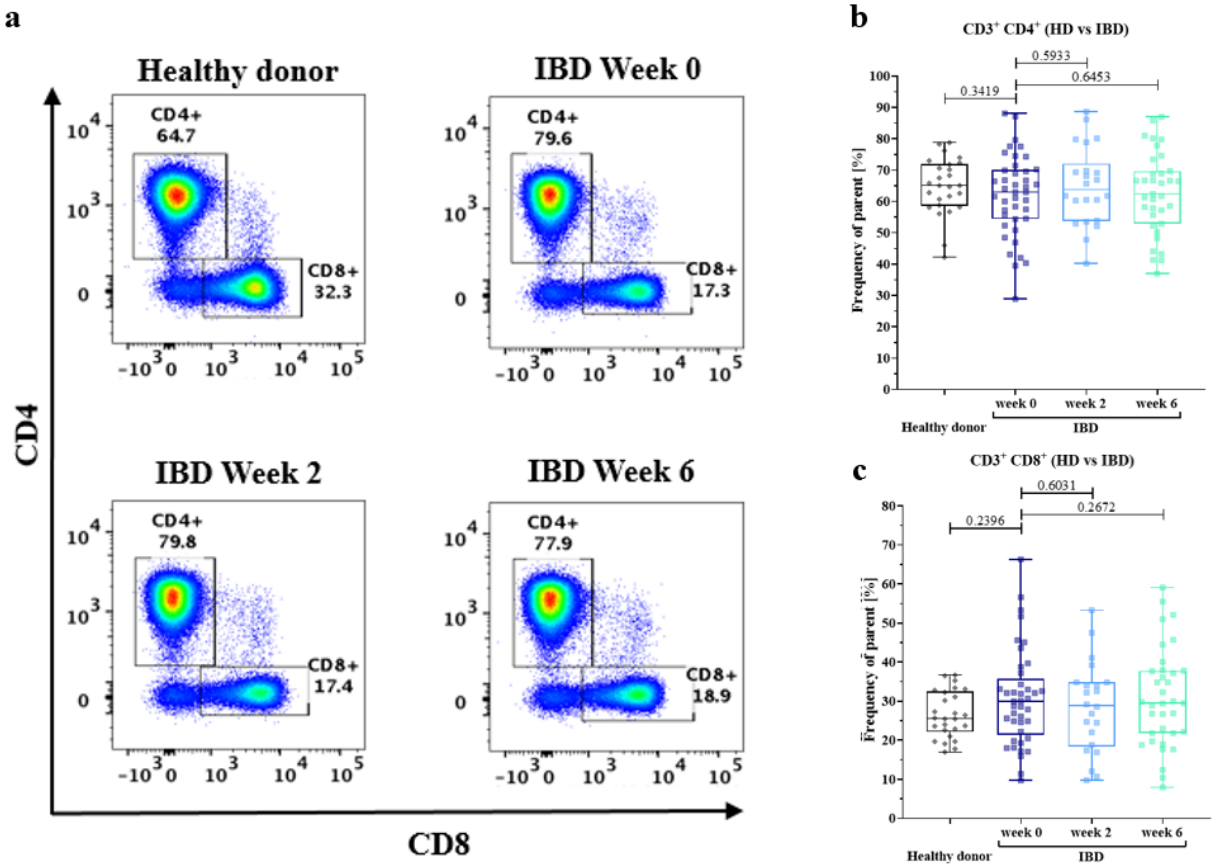


Figure 24: CD4$^+$ and CD8$^+$ T cells: Comparison of healthy donors and IBD patients

(gated on live cells -> single cells -> CD3$^+$)
***a**: Examples of flow cytometry plots showing the differences between healthy donors and the different time points*
***b**: Comparison of CD4$^+$ T cells in healthy donors and IBD patients (frequency of parent); n (HD) = 26; IBD: n (week 0) = 41; n (week 2) = 22; n (week 6) = 35; test used for HD vs IBD week 0: Mann-Whitney Test; test used for IBD week 0 vs week 2 and week 6: Wilcoxon Test*
***c**: Comparison of CD8$^+$ T cells in healthy donors and IBD patients; same cohort and tests used as in b*

Comparing $CD4^+$ and $CD8^+$ percentages in responders and non-responders, they showed different numbers on median: Responders were higher than non-responders in $CD4^+$ T cells at week 0 (65.3% vs. 60.2%; see Figure 25b). This shifted in week 2 (61.15% vs. 69.3%) and then turned back in week 6 (66.4% vs. 55.35%). This ratio was completely mirrored in $CD8^+$ T cells (week 0: 28.7% vs. 29%, week 2: 31.3% vs. 24.5%, week 6: 28.9% vs. 37%; see Figure 25c). However, no statistically significant distinction could be found between responders and non-responders in either $CD4^+$ or $CD8^+$ T cells.

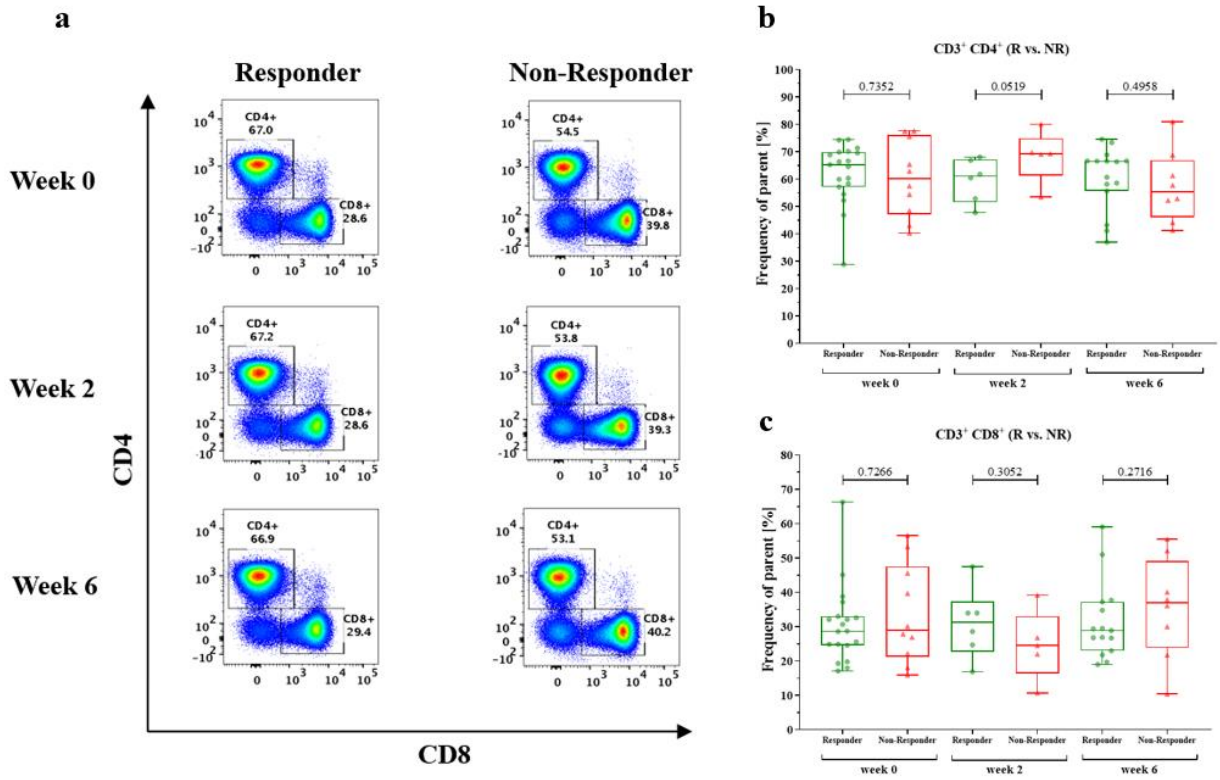


Figure 25: $CD4^+$ and $CD8^+$ T cells: Comparison of responders and non-responders

(gated on live cells -> single cells -> $CD3^+$)
***a**: Examples of flow cytometry plots showing the differences between responders and non-responders during the different weeks*
***b**: Comparison of $CD4^+$ T cells in responders and non-responders; responder: n (week 0) = 19; n (week 2) = 6; n (week 6) = 15; non-responder: n (week 0) = 10; n (week 2) = 5; n (week 6) = 8; test used: Mann-Whitney-Test*
***c**: Comparison of $CD8^+$ T cells in responders and non-responders; same cohort and tests used as in b*

4.3.4 Subsets in CD4$^+$ and CD8$^+$ T lymphocytes

Next, the major subsets of T lymphocytes – naïve T cells, memory T cells and regulatory T cells (T_{regs}) – were analysed in CD4$^+$ and CD8$^+$ T lymphocytes. Nevertheless, neither the comparison between healthy donors and IBD patients before therapy nor the changes between the different therapy time points turned out to be significant for any of these T cell subsets. They also did not show any predictive power regarding therapy outcome.

Looking at memory T cells in detail showed that IBD patients had higher percentage numbers at week 0 than healthy donors in both CD4$^+$ T lymphocytes (52.7% vs. 50.45%; see Figure 26a) and CD8$^+$ T lymphocytes (33.3% vs. 31.3%; see Figure 27a). The median level stays in both groups almost the same during therapy except for one spike from week 0 to week 2 in CD8$^+$ T lymphocytes (33.3% vs. 36.6%; see Figure 27a).

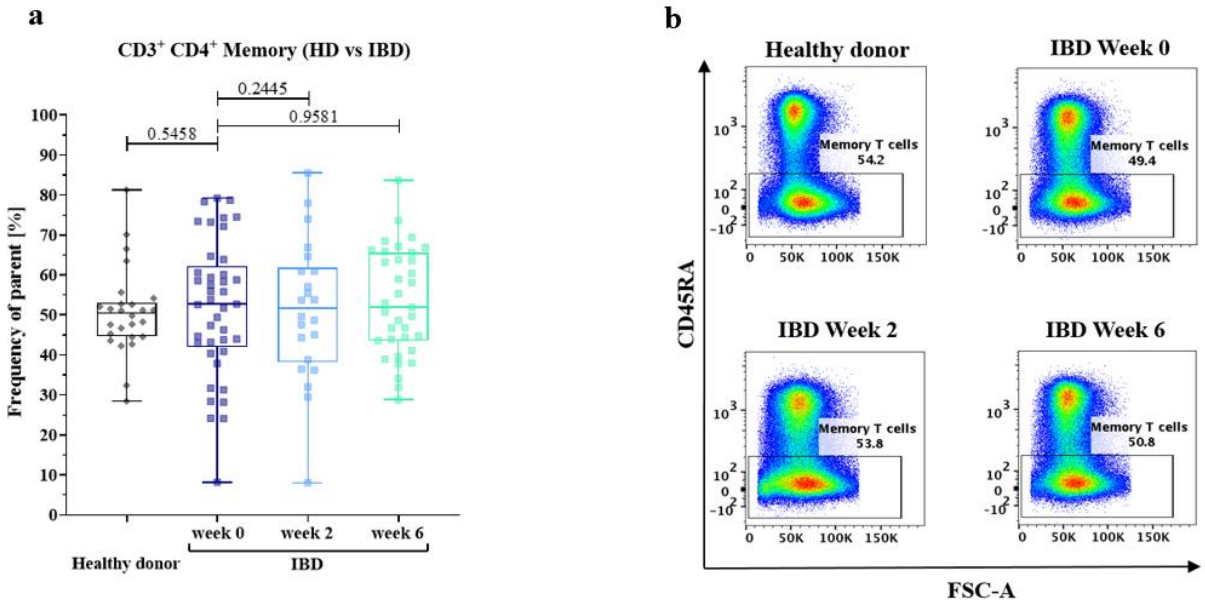


Figure 26: CD4$^+$ memory T cells: Comparison of healthy donors and IBD patients

(gated on live cells -> single cells -> CD3$^+$ -> CD4$^+$)
***a**: Comparison of healthy donors and IBD patients (frequency of parent); n (HD) = 26; IBD: n (week 0) = 41; n (week 2) = 22; n (week 6) = 35; test used for HD vs IBD week 0: Mann-Whitney Test; test used for IBD week 0 vs week 2 and week 6: Wilcoxon Test*
***b**: Examples of flow cytometry plots showing the differences between healthy donors and the different time points*

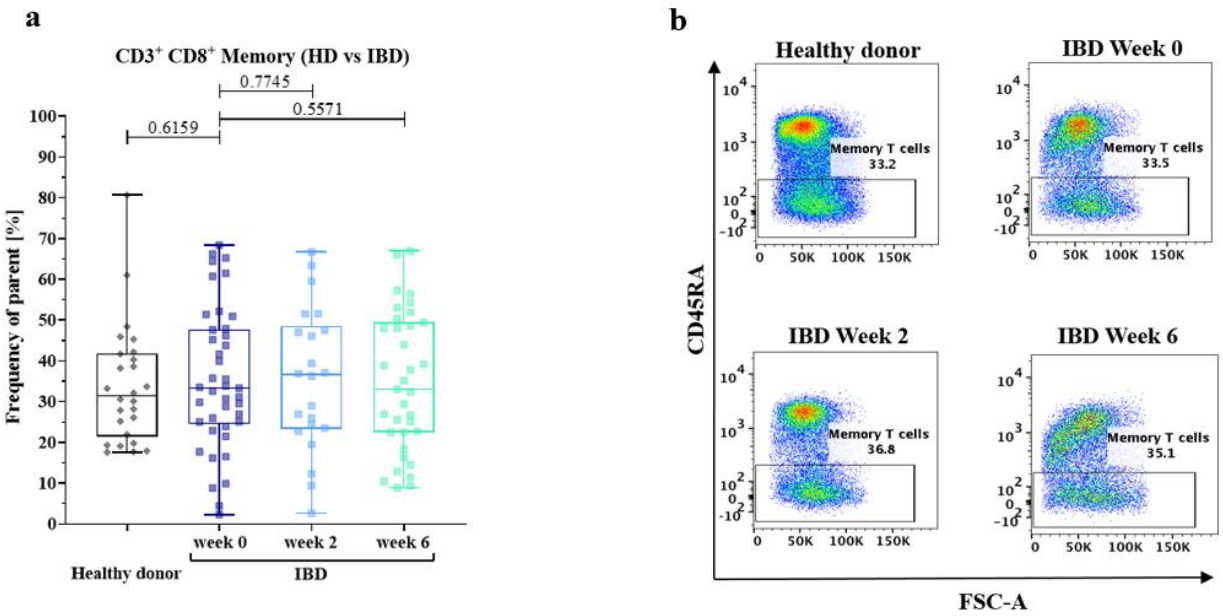


Figure 27: CD8+ memory T cells: Comparison of healthy donors and IBD patients

(gated on live cells -> single cells -> CD3+ -> CD8+)
***a**: Comparison of healthy donors and IBD patients (frequency of parent); n (HD) = 26; IBD: n (week 0) = 41; n (week 2) = 22; n (week 6) = 35; test used for HD vs IBD week 0: Mann-Whitney Test; test used for IBD week 0 vs week 2 and week 6: Wilcoxon Test*
***b**: Examples of flow cytometry plots showing the differences between healthy donors and the different time points*

Comparing responders and non-responders in $CD4^+$ memory T cells, non-responders are higher on a median level at week 0 (52.7% vs. 58.15%; see Figure 28a). This shifts in week 2 (60% vs. 53.7%) and turns back around in week 6 (53% vs. 62.95%). It is almost the same relation in $CD8^+$ memory T cells, except that at week 0 the median level is almost the same (week 0: 33.3% vs. 32.5%; week 2: 43.2% vs. 24.3%; week 6: 32.4% vs. 46.65%; see Figure 28c). However, as mentioned none of these comparisons are statistically significant.

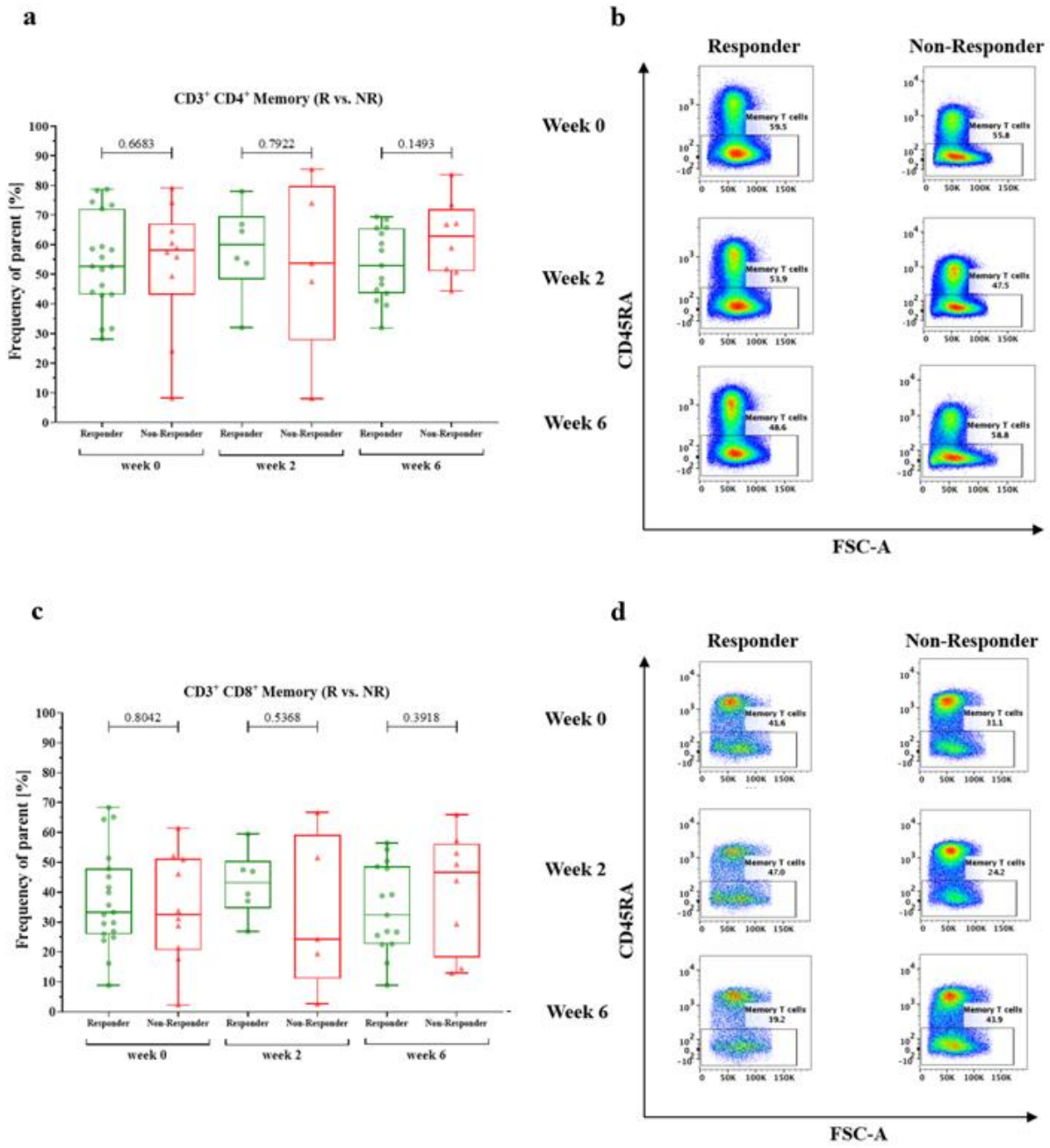


Figure 28: $CD4^+$ and $CD8^+$ memory T cells: Comparison of responders and non-responders

(gated on live cells -> single cells -> $CD3^+$ -> $CD4^+$ and $CD8^+$)
***a**: Comparison of responders and non-responders in $CD4^+$ memory T cells; responder: n (week 0) = 19; n (week 2) = 6; n (week 6) = 15; non-responder: n (week 0) = 10; n (week 2) = 5; n (week 6) = 8; test used: Mann-Whitney-Test*
***b**: Examples of flow cytometry plots showing the differences between responders and non-responders during the different week in $CD4^+$ memory T cells*
***c**: Comparison of responders and non-responders in $CD8^+$ memory T cells; same cohort and tests used as in a*
***d**: Examples of flow cytometry plots showing the differences between responders and non-responders during the different week in $CD8^+$ memory T cells*

The median level of $CD4^+$ and $CD8^+$ naïve T cells in IBD patients are both lower at week 0 compared to healthy donors ($CD4^+$: 43.4% vs. 49.7%; $CD8^+$: 34.2% vs. 50%; see Figure 29a and c). During therapy there are no major shifts in the median level. All comparisons stay statistically insignificant.

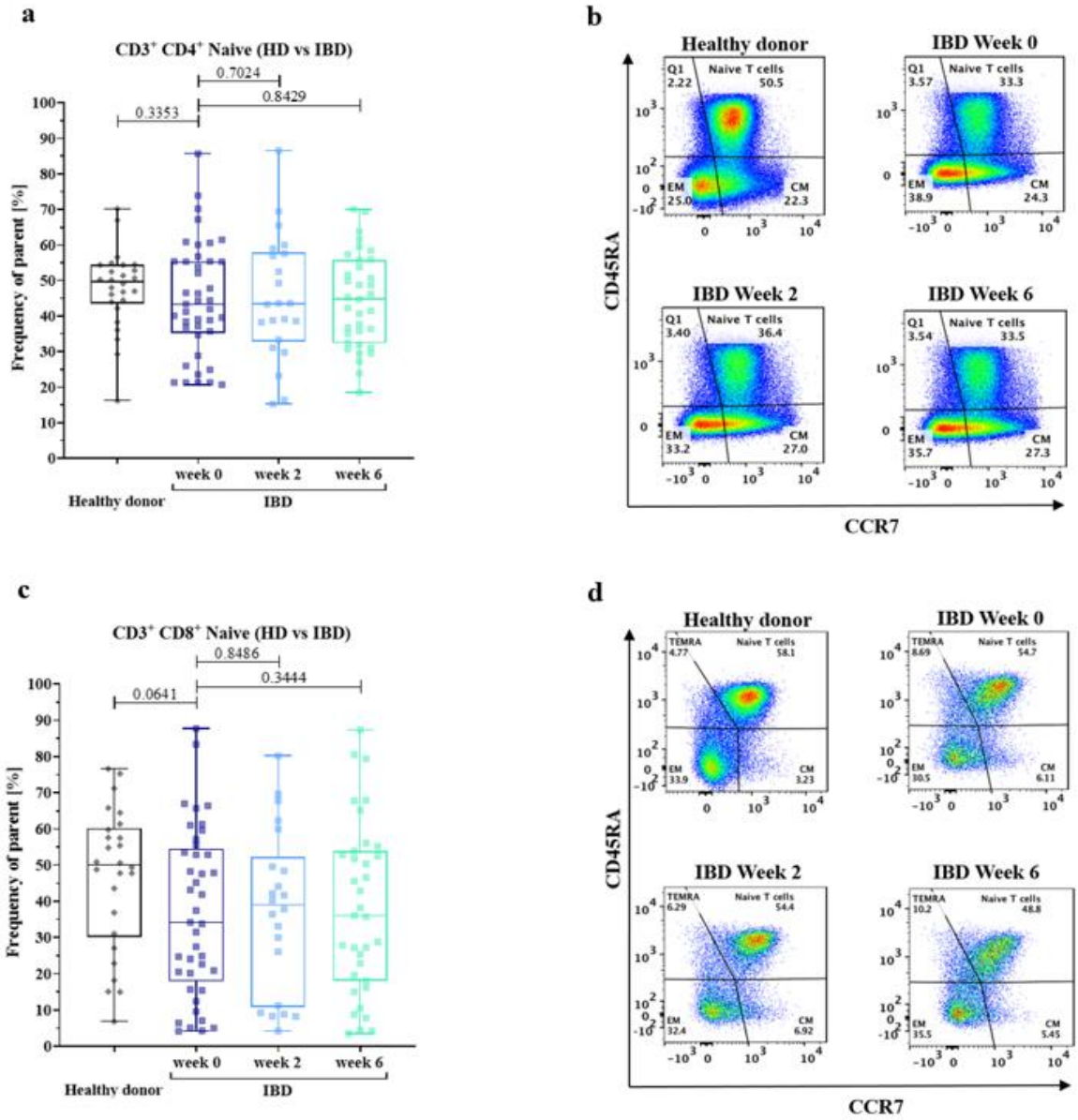


Figure 29: $CD4^+$ and $CD8^+$ naive T cells: Comparison of responders and non-responders

(gated on live cells -> single cells -> $CD3^+$ -> $CD4^+$ and $CD8^+$)
***a**: Comparison in healthy donors and IBD patients of $CD4^+$ naïve T cells (frequency of parent); n (HD) = 26; IBD: n (week 0) = 41; n (week 2) = 22; n (week 6) = 35; test used for HD vs IBD week 0: Mann-Whitney Test; test used for IBD week 0 vs week 2 and week 6: Wilcoxon Test*
***b**: Examples of flow cytometry plots showing the differences of $CD4^+$ naïve T cells between healthy donors and the different time points*
***c**: Comparison in healthy donors and IBD patients of $CD8^+$ naïve T cells (frequency of parent); ; same cohort and tests used as in a*
***d**: Examples of flow cytometry plots showing the differences of $CD8^+$ naïve T cells between healthy donors and the different time points*

An opposite trend compared to memory T cells can be detected in naïve T cells. In $CD4^+$ naïve T cells responders have the higher median than non-responders at week 0 (44.4% vs. 39.6%; see Figure 30a). It switches at week 2 (37.35% vs. 43,3%) and turns back around at week 6 (46.2% vs. 35.95%). For memory T cells this was the other way around. In $CD8^+$ naïve T cells the same trend can be detected, except for week 0 at which the median is almost the same in responders and non-responders (34.2% vs. 35.05%; see Figure 31a).

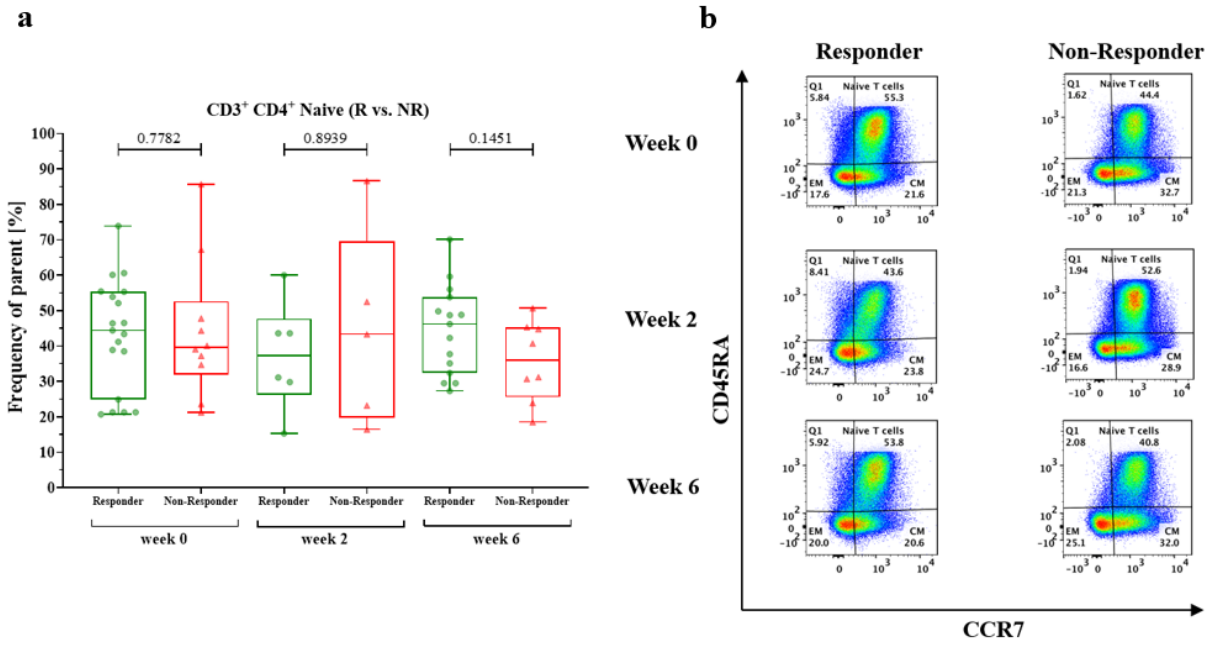


Figure 30: $CD4^+$ naive T cells: Comparison of responders and non-responders

***a**: Comparison of responders and non-responders; responder: n (week 0) = 19; n (week 2) = 6; n (week 6) = 15; non-responder: n (week 0) = 10; n (week 2) = 5; n (week 6) = 8; test used: Mann-Whitney-Test*
***b**: Examples of flow cytometry plots showing the differences between responders and non-responders during the different weeks*

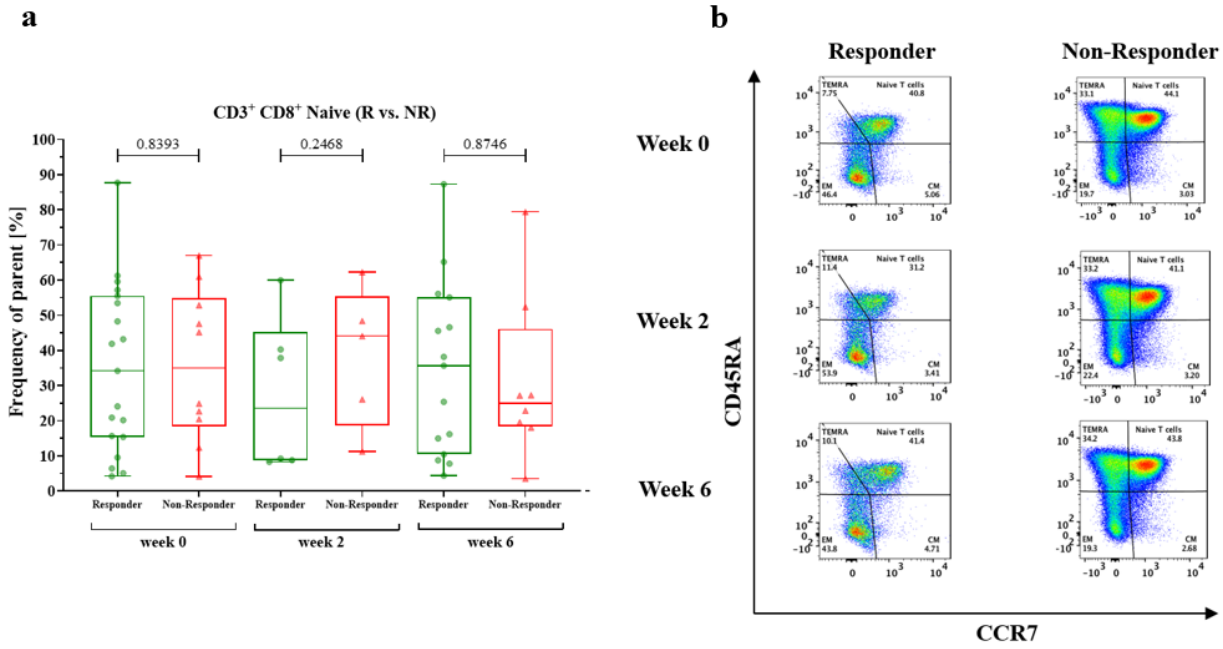


Figure 31: CD8$^+$ naive T cells: Comparison of responders and non-responders

***a**: Comparison of responders and non-responders; responder: n (week 0) = 19; n (week 2) = 6; n (week 6) = 15; non-responder: n (week 0) = 10; n (week 2) = 5; n (week 6) = 8; test used: Mann-Whitney-Test*
***b**: Examples of flow cytometry plots showing the differences between responders and non-responders during the different weeks*

The CD4$^+$ T_{regs} are on a median level lower in IBD patients at week 0 than in healthy donors (1.23% vs. 1.49%; see Figure 32a). The median level drops in week 2 to 0.86% and recovers back to 1.18% in week 6. Looking at the difference between responders and non-responders the median level is higher in week 0 and week 2 in non-responders (week 0: 1.21% vs. 1.66%; week 2: 0.53% vs. 1.24%; see Figure 32c). This turns around in week 6 (1.27% vs. 1.05%). Nevertheless, none of the comparisons show a statistically significant difference.

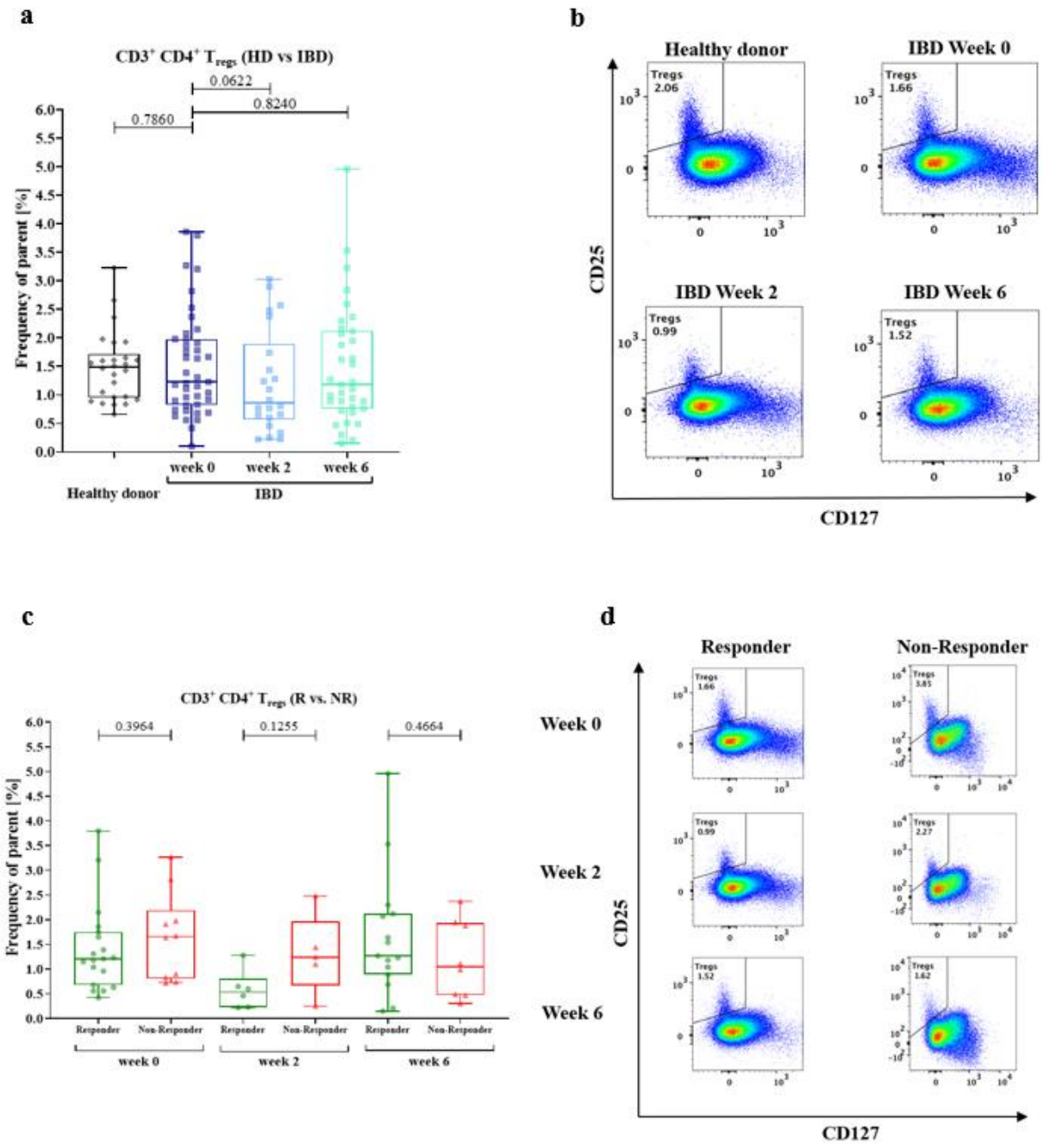


Figure 32: CD4+ regulatory T cells

(gated on live cells -> single cells -> CD3+ -> CD4+)
***a**: Comparison of healthy donors and IBD patients (frequency of parent); n (HD) = 26; IBD: n (week 0) = 41; n (week 2) = 22; n (week 6) = 35; test used for HD vs IBD week 0: Mann-Whitney Test; test used for IBD week 0 vs week 2 and week 6: Wilcoxon Test*
***b**: Examples of flow cytometry plots showing the differences between healthy donors and the different time points*
***c**: Comparison of responders and non-responders; responder: n (week 0) = 19; n (week 2) = 6; n (week 6) = 15; non-responder: n (week 0) = 10; n (week 2) = 5; n (week 6) = 8; test used: Mann-Whitney-Test*
***d**: Examples of flow cytometry plots showing the differences between responders and non-responders during the different weeks*

The results for the subgroups of memory T cells – central and effector memory T cells (T_{CM} and T_{EM}) – turned out similar: In both subgroups, no significant difference could be found concerning the research questions.

The $CD4^+$ T_{CM} showed no major differences between healthy donors and IBD patients at baseline nor during therapy compared to week 0 (see Figure 33b). For $CD4^+$ T_{EM} the median levels looked similar except for the median in IBD patients being higher at week 0 than in healthy donors (24.9% vs. 18.85%; see Figure 33c). As mentioned above, these ratios do not show statistically significant difference.

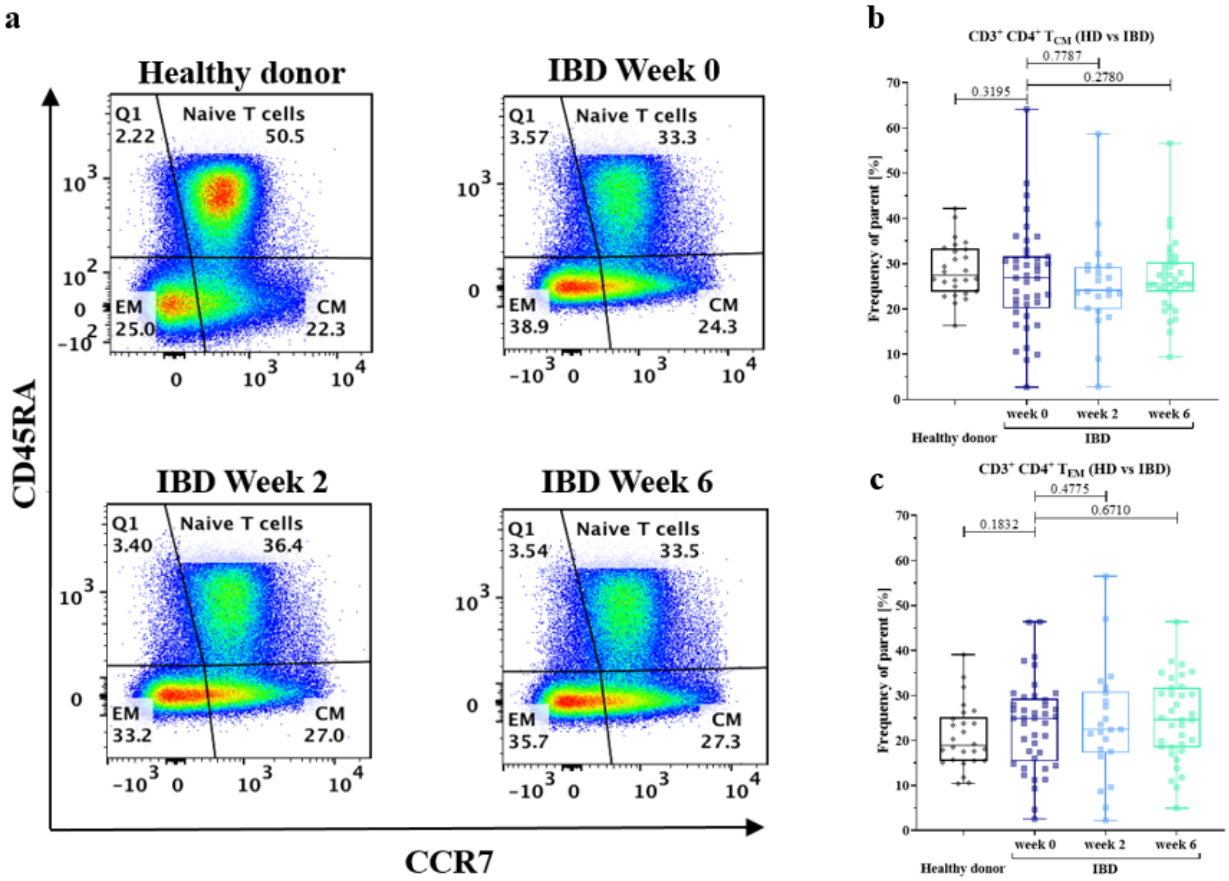


Figure 33: $CD4^+$ central and effector memory T cells: Comparison of healthy donors and IBD patients

(gated on live cells -> single cells -> $CD3^+$ -> $CD4^+$)
***a**: Examples of flow cytometry plots showing the differences between healthy donors and the different time points*
***b**: Comparison of $CD4^+$ T_{CM} in healthy donors and IBD patients (frequency of parent); n (HD) = 26; IBD: n (week 0) = 41; n (week 2) = 22; n (week 6) = 35; test used for HD vs IBD week 0: Mann-Whitney Test; test used for IBD week 0 vs week 2 and week 6: Wilcoxon Test*
***c**: Comparison of $CD4^+$ effector memory T cells in healthy donors and IBD patients; same cohort and tests used as in b*

The median of $CD4^+$ T_{CM} is through the three timepoints always higher in non-responders than responders (see Figure 34b). $CD4^+$ T_{EM} tend to have similar median levels in responders and non-responders except for week 2 in which responders´ median is at 29% compared to 23.5% in non-responders (see Figure 34c). No group comparison turned out to be statistically significant.

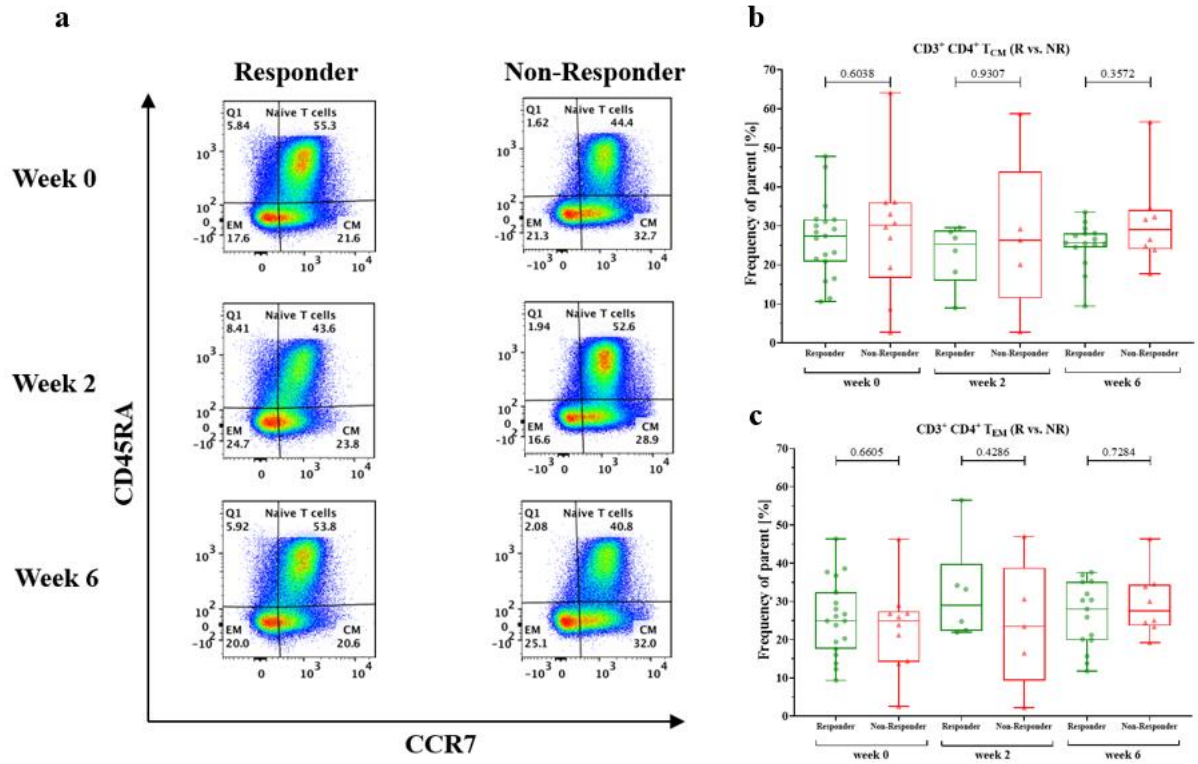


Figure 34: $CD4^+$ central and effector memory T cells: Comparison of responders and non-responders

(gated on live cells -> single cells -> $CD3^+$ -> $CD4^+$)
***a**: Examples of flow cytometry plots showing the differences between responders and non-responders during the different weeks*
***b**: Comparison of $CD4^+$ T_{CM} in responders and non-responders; responder: n (week 0) = 19; n (week 2) = 6; n (week 6) = 15; non-responder: n (week 0) = 10; n (week 2) = 5; n (week 6) = 8; test used: Mann-Whitney-Test*
***c**: Comparison of $CD4^+$ effector memory T cells in responders and non-responders; same cohort and tests used as in b*

The CD8$^+$ T_{CM} show like the CD4$^+$ T_{CM} no major or significant differences between healthy donors at IBD patients at baseline nor compared during therapy (see Figure 35b). The median of CD8$^+$ T_{EM} in IBD patients at baseline is lower than in healthy donors (34.5% vs. 36.7%; see Figure 35c). During therapy in than rises to 36.55% and 38.8% in week 6.

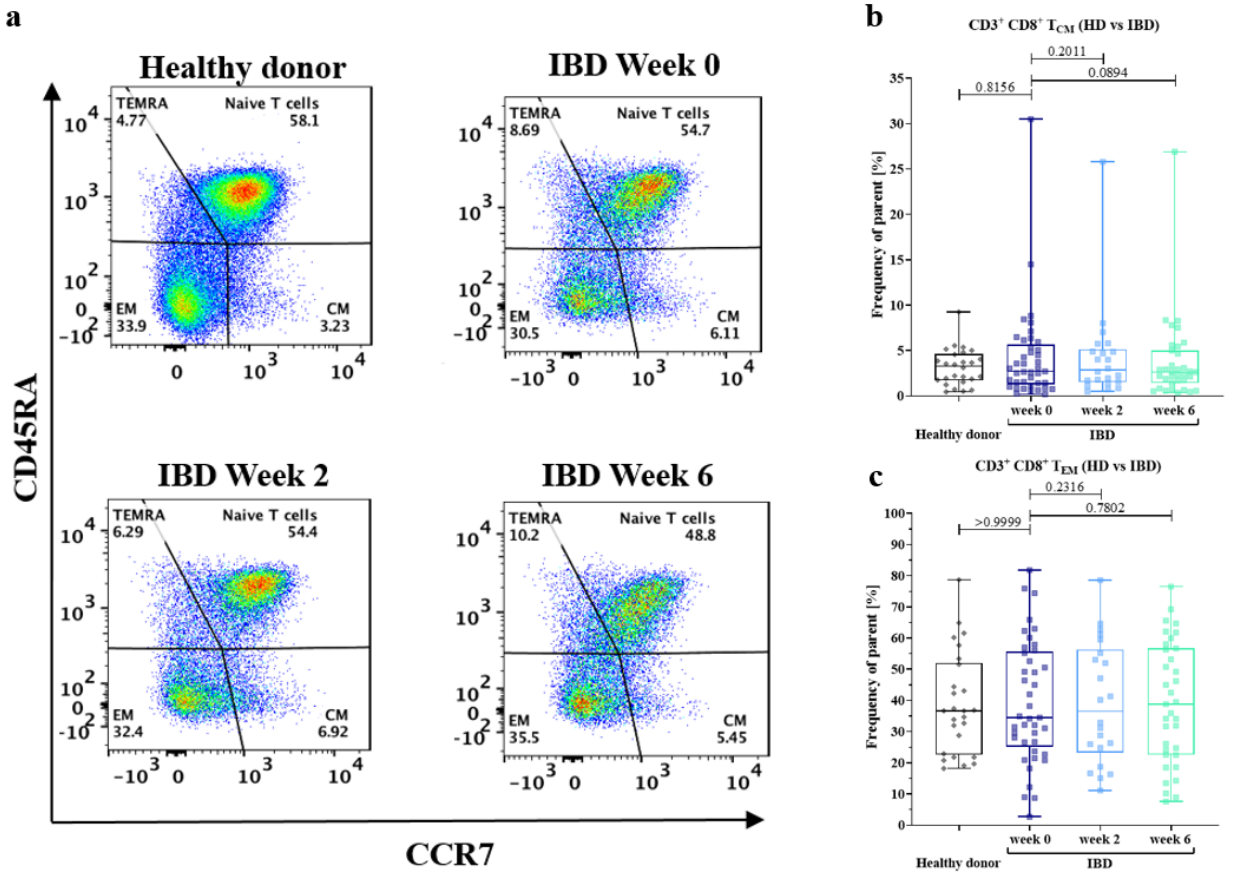


Figure 35: CD8$^+$ central and effector memory T cells: Comparison of healthy donors and IBD patients

(gated on live cells -> single cells -> CD3$^+$ -> CD8$^+$)
***a**: Examples of flow cytometry plots showing the differences between healthy donors and the different time points*
***b**: Comparison of CD8$^+$ T_{CM} in healthy donors and IBD patients (frequency of parent); n (HD) = 26; IBD: n (week 0) = 41; n (week 2) = 22; n (week 6) = 35; test used for HD vs IBD week 0: Mann-Whitney Test; test used for IBD week 0 vs week 2 and week 6: Wilcoxon Test*
***c**: Comparison of CD8$^+$ effector memory T cells in healthy donors and IBD patients; same cohort and tests used as in b*

Comparing the median levels in responders and non-responders shows a mixed picture: While responders have a higher median in $CD8^+$ T_{CM} at week 0, non-responders´ median is higher in the rest of the induction phase (see Figure 36b). The baseline median of $CD8^+$ T_{EM} is similar in responders and non-responders, while in week 2 responders and in week 6 non-responders show a higher median (see Figure 36c). However, all these comparisons are statistically insignificant.

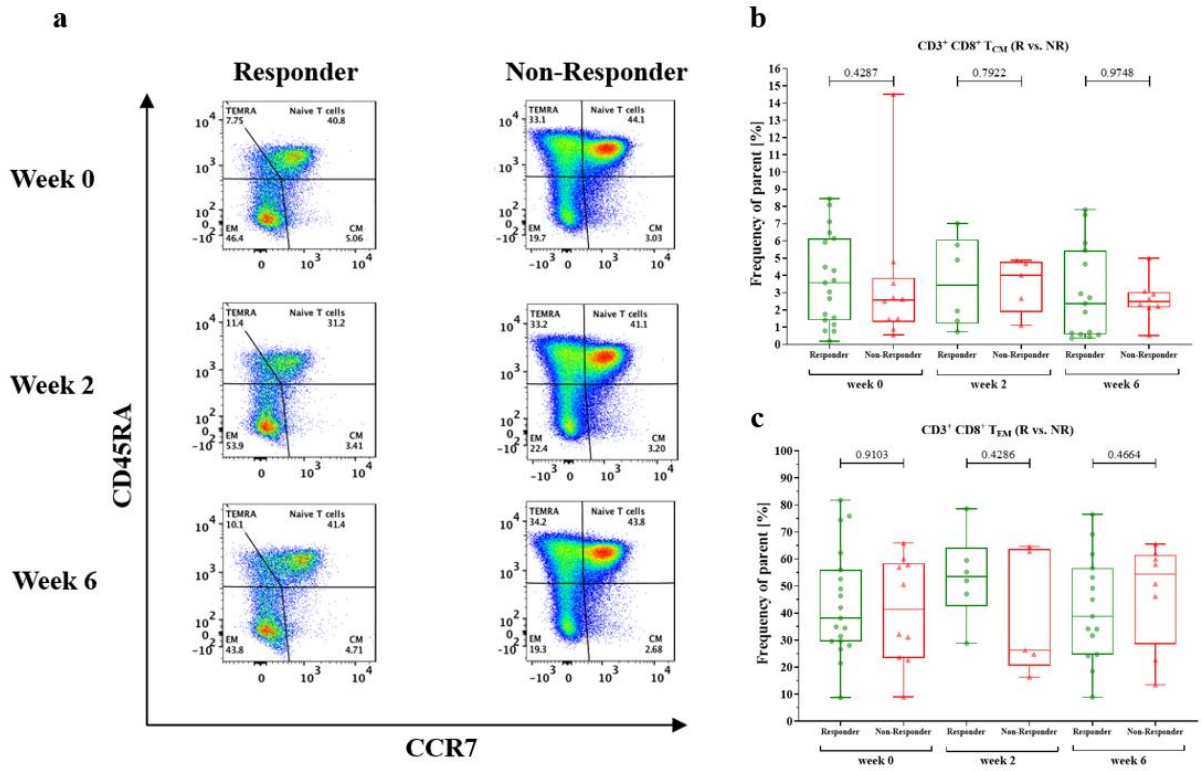


Figure 36: $CD8^+$ central and effector memory T cells: Comparison of responders and non-responders

(gated on live cells -> single cells -> $CD3^+$ -> $CD8^+$)
***a**: Examples of flow cytometry plots showing the differences between responders and non-responders during the different weeks*
***b**: Comparison of $CD8^+$ T_{CM} in responders and non-responders; responder: n (week 0) = 19; n (week 2) = 6; n (week 6) = 15; non-responder: n (week 0) = 10; n (week 2) = 5; n (week 6) = 8; test used: Mann-Whitney-Test*
***c**: Comparison of $CD8^+$ effector memory T cells in responders and non-responders; same cohort and tests used as in b*

For CD8$^+$ T_{EMRA} a significantly higher percentage could be found in IBD patients before therapy in comparison to healthy donors (median: 10.8% vs. 8.1%; see Figure 37b). The absolute cell numbers showed the same trend, even though it was not significant (see Figure 37c). No statistically significant difference could be found comparing week 2 and week 6 to baseline.

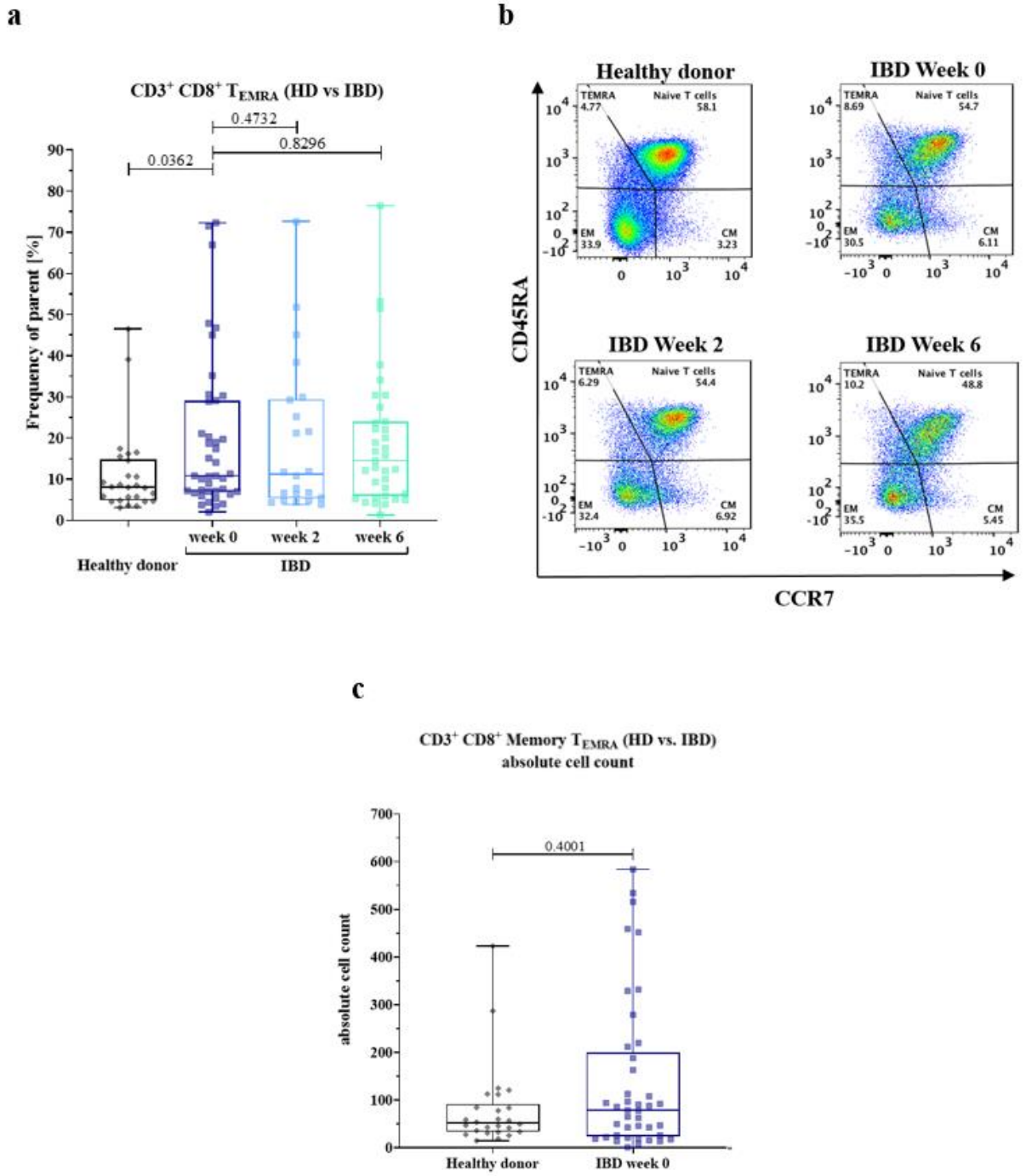


Figure 37: CD8$^+$ TEMRA cells: Comparison of healthy donors and IBD patients

(gated on live cells -> single cells -> CD3$^+$ -> CD8$^+$)
***a**: Comparison of healthy donors and IBD patients (frequency of parent); n (HD) = 26; IBD: n (week 0) = 41; n (week 2) = 22; n (week 6) = 35; test used for HD vs IBD week 0: Mann-Whitney Test; test used for IBD week 0 vs week 2 and week 6: Wilcoxon Test*
***b**: Examples of flow cytometry plots showing the differences between healthy donors and the different time points*
***c**: Comparison of healthy donors and IBD patients at week 0 (absolute cell count); same cohort and tests used as in a*

This CD8$^+$ subcluster also did not turn out to be predictive for therapy response, even though the median of non-responders was higher during all three timepoints (week 0: 11.3% vs, 17.05%; week 2: 11.8% vs. 21.2%; week 6: 14.5% vs. 20.55%; see Figure 38b).

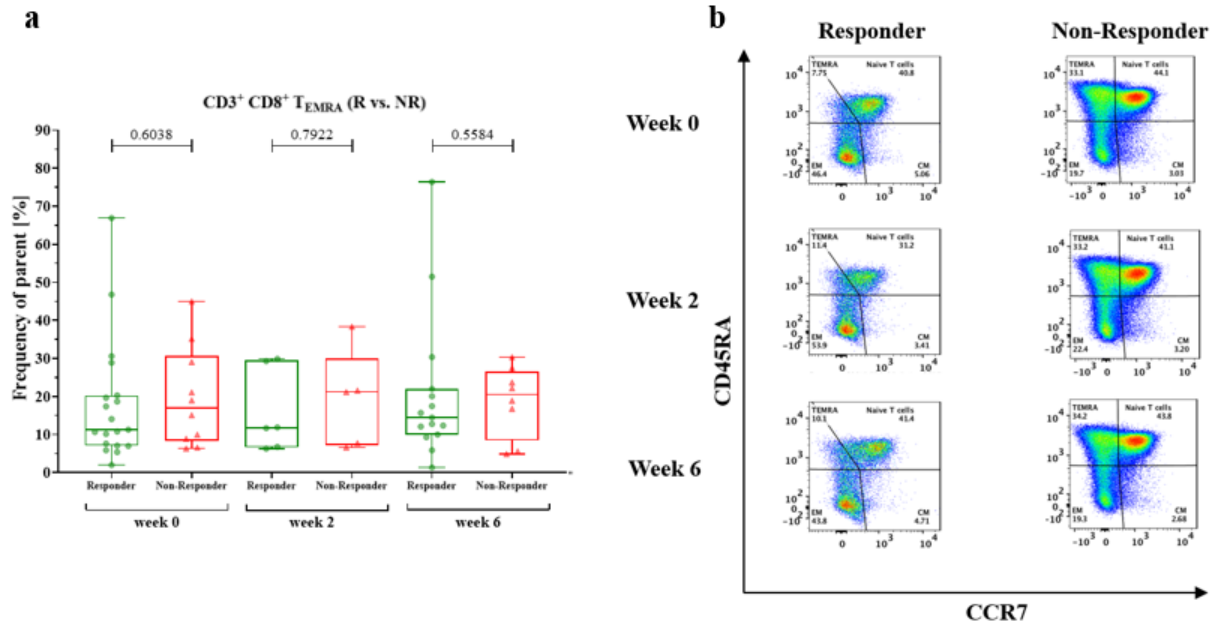


Figure 38: CD8$^+$ TEMRA cells: Comparison of responders and non-responders

(gated on live cells -> single cells -> CD3$^+$ -> CD8$^+$)
***a**: Comparison of responders and non-responders; responder: n (week 0) = 19; n (week 2) = 6; n (week 6) = 15; non-responder: n (week 0) = 10; n (week 2) = 5; n (week 6) = 8; test used: Mann-Whitney-Test*
***b**: Examples of flow cytometry plots showing the differences between responders and non-responders during the different weeks*

4.3.5 Gut-homing markers on $CD4^+$ and $CD8^+$ memory T cells

Next, I looked at two major gut-homing markers besides the integrins which are CCR9 and GPR15. Both have been discussed to play a major role in IBD for immune cells to enter the gut tissue (98,99). In my cohort, GPR15 is statistically significantly increased on both $CD4^+$ and $CD8^+$ memory T cells in percentage numbers when comparing IBD patients before therapy to healthy donors. In $CD4^+$ memory T cells the median was at 9.93% in IBD patients at baseline compared to 7.35% in healthy donors (see Figure 39a).

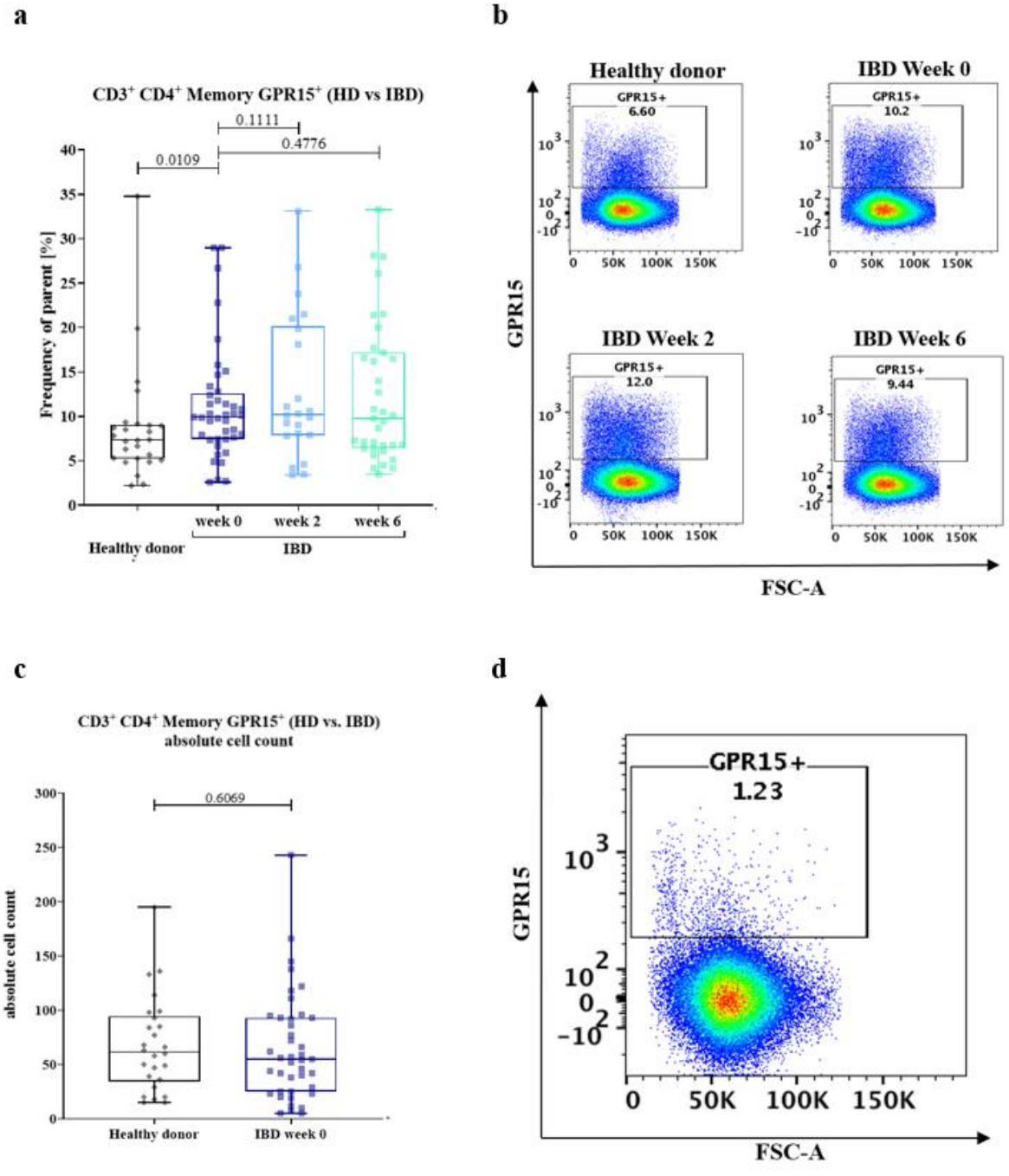


Figure 39:GPR15-expression on CD4$^+$ memory T cells

(gated on live cells -> single cells -> CD3$^+$ -> CD4$^+$ -> CD45RA$^-$)
***a**: Comparison of healthy donors and IBD patients (frequency of parent); n (HD) = 26; IBD: n (week 0) = 41; n (week 2) = 22; n (week 6) = 35; test used for HD vs IBD week 0: Mann-Whitney Test; test used for IBD week 0 vs week 2 and week 6: Wilcoxon Test*
***b**: Examples of flow cytometry plots showing the differences between healthy donors and the different time points*
***c**: Comparison of healthy donors and IBD patients at week 0 (absolute cell count); same cohort and tests used as in a*
***d:** Control of GPR15-staining on naïve CD4$^+$ T cells, which show no expression*

In CD8$^+$ memory T cells the difference was even bigger with 8.73% vs, 4.8%. (see Figure 40a) However, this statistically significant difference could not be shown in absolute cell numbers (see Figure 39c and Figure 41). Throughout the therapy, the numbers do not change in a significant way.

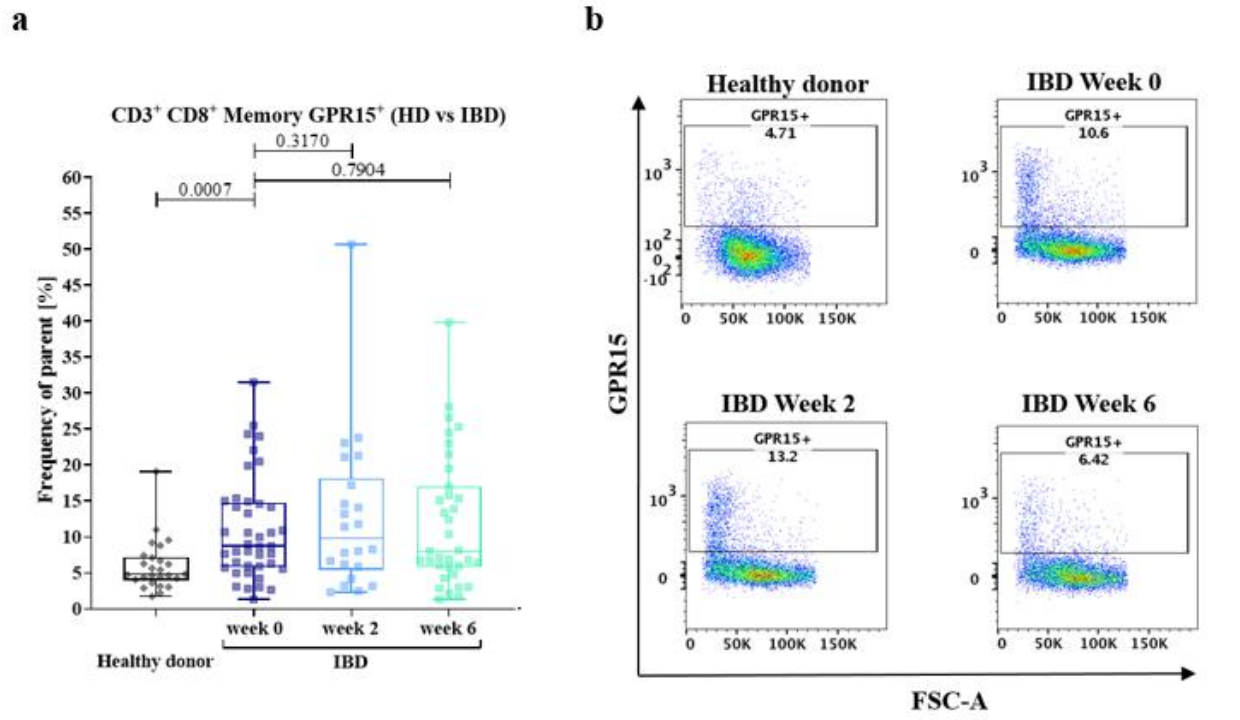


Figure 40: GPR15-expression on CD8$^+$ memory T cells: Comparison of healthy donors and IBD patients

(gated on live cells -> single cells -> CD3$^+$ -> CD8$^+$ -> CD45RA$^-$)
***a**: Comparison of healthy donors and IBD patients (frequency of parent); n (HD) = 26; IBD: n (week 0) = 41; n (week 2) = 22; n (week 6) = 35; test used for HD vs IBD week 0: Mann-Whitney Test; test used for IBD week 0 vs week 2 and week 6: Wilcoxon Test*
***b**: Examples of flow cytometry plots showing the differences between healthy donors and the different time points*

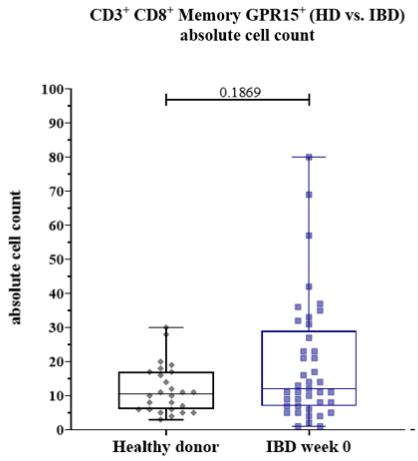


Figure 41: GPR15-expression on CD8$^+$ memory T cells: Comparison of healthy donors and IBD patients (absolute cell count)

(gated on live cells -> single cells -> CD3$^+$ -> CD8$^+$ -> CD45RA$^-$)
Comparison of healthy donors and IBD patients (absolute cell count); n (HD) = 26; IBD: n (week 0) = 41; test used for HD vs IBD week 0: Mann-Whitney Test

Also, GPR15$^+$ expression on CD4$^+$ or CD8$^+$ memory T cells did not predict therapy outcome. In CD4$^+$ memory the median levels are always quite similar except for week 6 in which non-responders tend to have a higher median (9.48% vs. 11.24%; see Figure 42a). The difference is even bigger in CD8$^+$ memory T cells not only at week 6 (6.92% vs. 15.25%) but also at week 2 (12.3% vs. 14.6%; see Figure 42c).

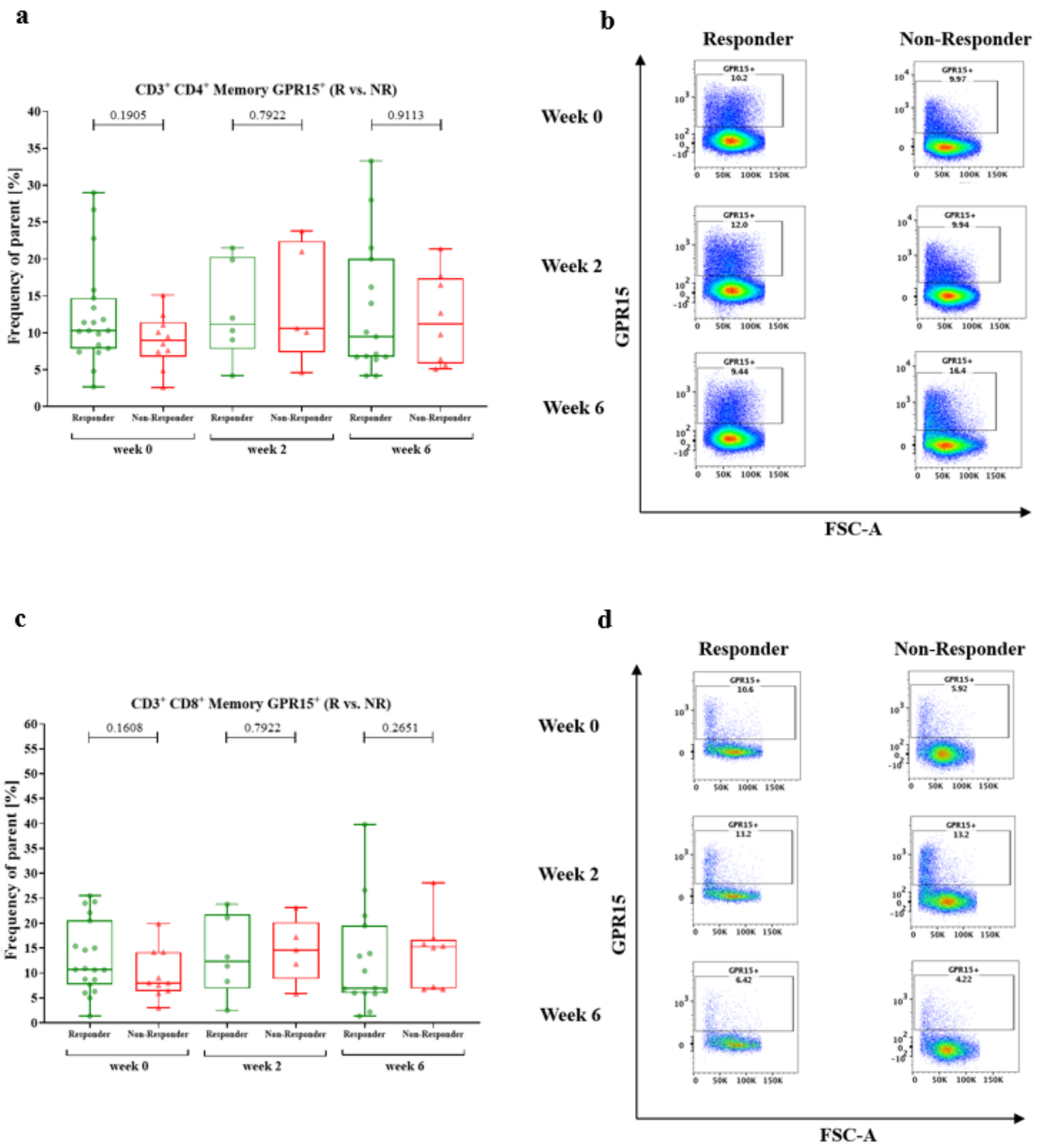


Figure 42: GPR15-expression on $CD4^+$ and $CD8^+$ memory T cells: Comparison of responders and non-responders

(gated on live cells -> single cells -> $CD3^+$ -> $CD4^+$ and $CD8^+$ -> $CD45RA^-$)
***a**: Comparison of GP15-expression on $CD4^+$ memory T cells in responders and non-responders; responder: n (week 0) = 19; n (week 2) = 6; n (week 6) = 15; non-responder: n (week 0) = 10; n (week 2) = 5; n (week 6) = 8; test used: Mann-Whitney-Test*
***b**: Examples of flow cytometry plots showing the differences of GP15-expression on $CD4^+$ memory T cells between responders and non-responders during the different weeks*
***c**: Comparison of GP15-expression on $CD8^+$ memory T cells in responders and non-responders; same cohort and tests used as in a*
***d**: Examples of flow cytometry plots showing the differences of GP15-expression on $CD8^+$ memory T cells between responders and non-responders during the different weeks*

Comparing CCR9 expression on $CD4^+$ memory T cells in healthy donors and IBD patients before therapy, a statistically significant difference in relative numbers could be shown with 7.73% in IBD patients at baseline and 3.88% in healthy donors on median (see Figure 43a). In absolute number this same significance could not be verified (see Figure 43c). During vedolizumab induction the median of CCR9-expression decreases on median but not statistically significant.

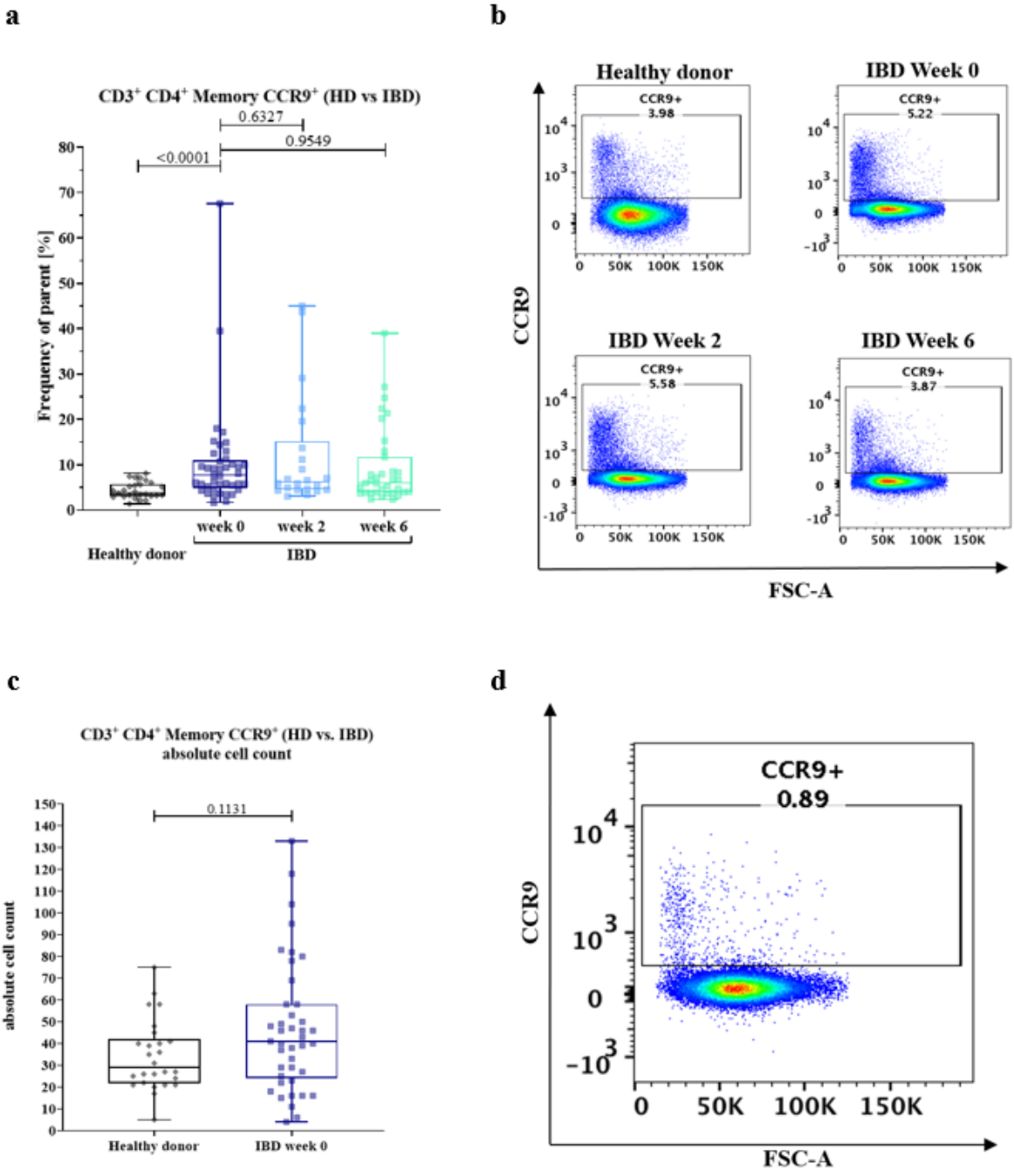


Figure 43: CCR9-expression on $CD4^+$ memory T cells: Comparison of healthy donors and IBD patients

(gated on live cells -> single cells -> $CD3^+$ -> $CD4^+$ -> $CD45RA^-$)
***a**: Comparison of healthy donors and IBD patients (frequency of parent); n (HD) = 26; IBD: n (week 0) = 41; n (week 2) = 22; n (week 6) = 35; test used for HD vs IBD week 0: Mann-Whitney Test; test used for IBD week 0 vs week 2 and week 6: Wilcoxon Test*
***b**: Examples of flow cytometry plots showing the differences between healthy donors and the different time points*
***c**: Comparison of healthy donors and IBD patients at week 0 (absolute cell count); same cohort and tests used as in a*
***d:** Control of CCR9-staining on naïve $CD4^+$ T cells, which show no expression*

A similar expression pattern can be seen in $CD8^+$ memory T cells: While the comparison of relative numbers between IBD patients at baseline and healthy donors is statistically significant (median: 9.77% vs. 4.07%; see Figure 44a), the absolute numbers are not statistically different (see Figure 44c). During therapy, the median decreases as well but not statistically significantly (week 2: 7.1%; week: 6.99%).

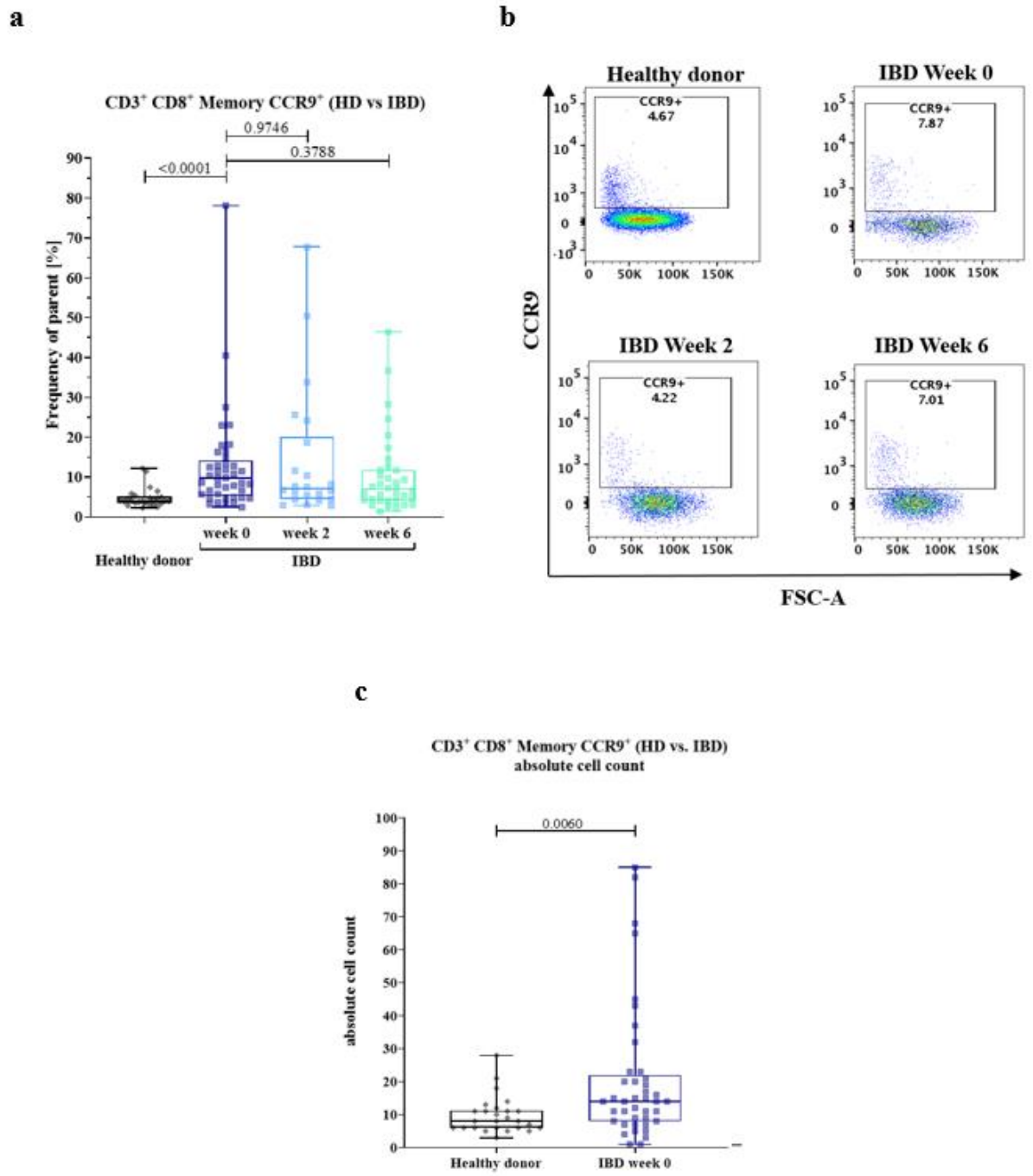


Figure 44: CCR9-expression on CD8$^+$ memory T cells: Comparison of healthy donors and IBD patients

(gated on live cells -> single cells -> CD3$^+$ -> CD8$^+$ -> CD45RA$^-$)
***a**: Comparison of healthy donors and IBD patients (frequency of parent); n (HD) = 26; IBD: n (week 0) = 41; n (week 2) = 22; n (week 6) = 35; test used for HD vs IBD week 0: Mann-Whitney Test; test used for IBD week 0 vs week 2 and week 6: Wilcoxon Test*
***b**: Examples of flow cytometry plots showing the differences between healthy donors and the different time points*
***c**: Comparison of healthy donors and IBD patients at week 0 (absolute cell count); same cohort and tests used as in a*

Comparing CCR9-expression in responders and non-responders for predictive power shows no statistically significant difference in either $CD4^+$ nor in $CD8^+$ memory T cells: The median of CCR9-expression on $CD4^+$ memory T cells in responders is higher than in non-responders at baseline and week 2 (see Figure 45a). This changes in week 6 to. This ratio is similar in $CD8^+$ memory T cells (see Figure 46a).

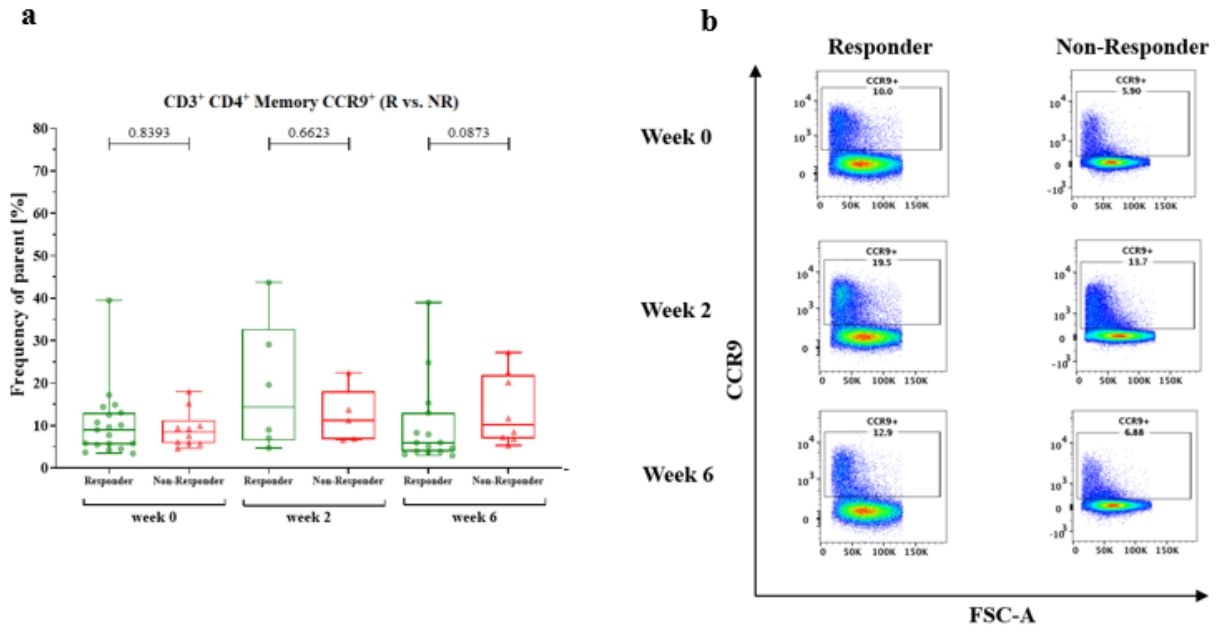


Figure 45: CCR9-expression on $CD4^+$ memory T cells: Comparison of responders and non-responders

(gated on live cells -> single cells -> $CD3^+$ -> $CD4^+$ -> $CD45RA^-$)
***a**: Comparison of CCR9-expression on $CD4^+$ memory T cells in responders and non-responders; responder: n (week 0) = 19; n (week 2) = 6; n (week 6) = 15; non-responder: n (week 0) = 10; n (week 2) = 5; n (week 6) = 8; test used: Mann-Whitney-Test*
***b**: Examples of flow cytometry plots showing the differences of CCR9-expression on $CD4^+$ memory T cells between responders and non-responders during the different weeks*

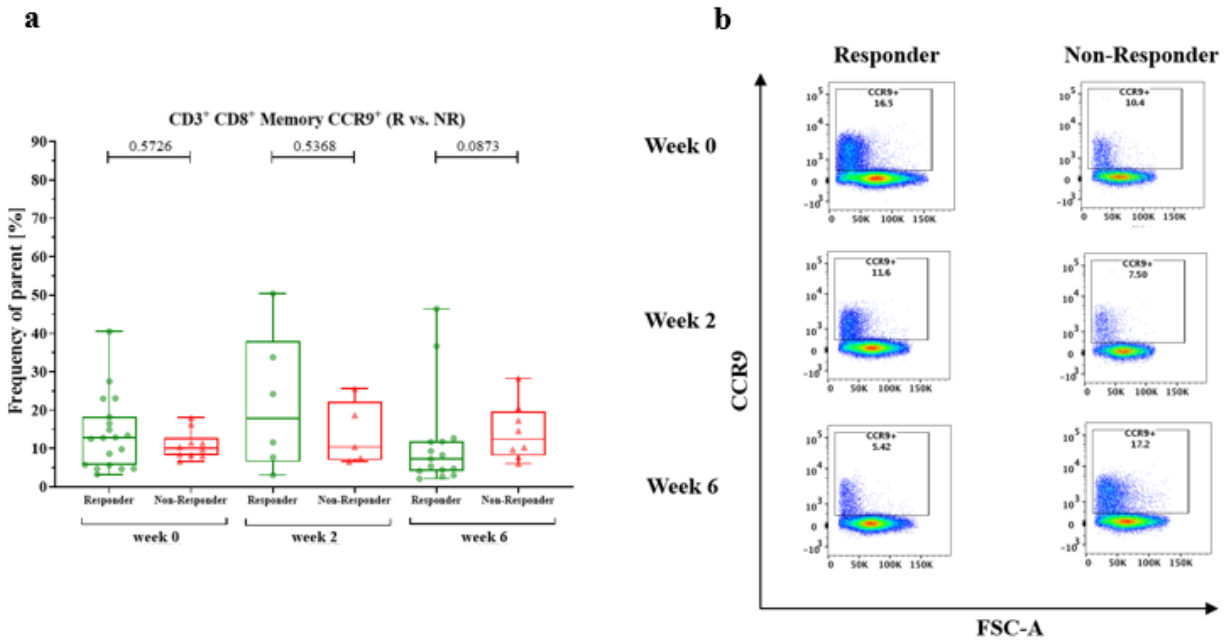


Figure 46: CCR9-expression on CD8$^+$ memory T cells: Comparison of responders and non-responders

(gated on live cells -> single cells -> CD3$^+$ -> CD8$^+$ -> CD45RA$^-$)
***a**: Comparison of CCR9-expression on CD8$^+$ memory T cells in responders and non-responders; responder: n (week 0) = 19; n (week 2) = 6; n (week 6) = 15; non-responder: n (week 0) = 10; n (week 2) = 5; n (week 6) = 8; test used: Mann-Whitney-Test*
***b**: Examples of flow cytometry plots showing the differences of CCR9-expression on CD8$^+$ memory T cells between responders and non-responders during the different weeks*

4.3.6 Integrin markers on CD4$^+$ and CD8$^+$ memory T cells

The next topic to look at was the integrins. In particular, the expression of α4-integrin and β7-integrin was interesting, as it is the target of vedolizumab. But α4 can also build a heterodimer with β1 and β7 can bind with αE. So, I analysed all their different combination possibilities to investigate, if there were any changes under vedolizumab treatment. Because the integrins are mostly expressed on memory T cells (51), I focused on this subgroup.

The combined expression of α4β7-Integrin was statistically significantly lower in IBD patients before therapy in comparison to healthy donors in both CD4$^+$ memory T cells (median: 12.4% vs. 16.35%; see Figure 47a) and CD8$^+$ memory T cells (median: 18.2% vs. 28.9%; see Figure 48a). This could be shown in relative and absolute numbers (see Figure 47c and Figure 48c). During therapy it statistically significantly decreased already after week 2 and stayed low in week 6.

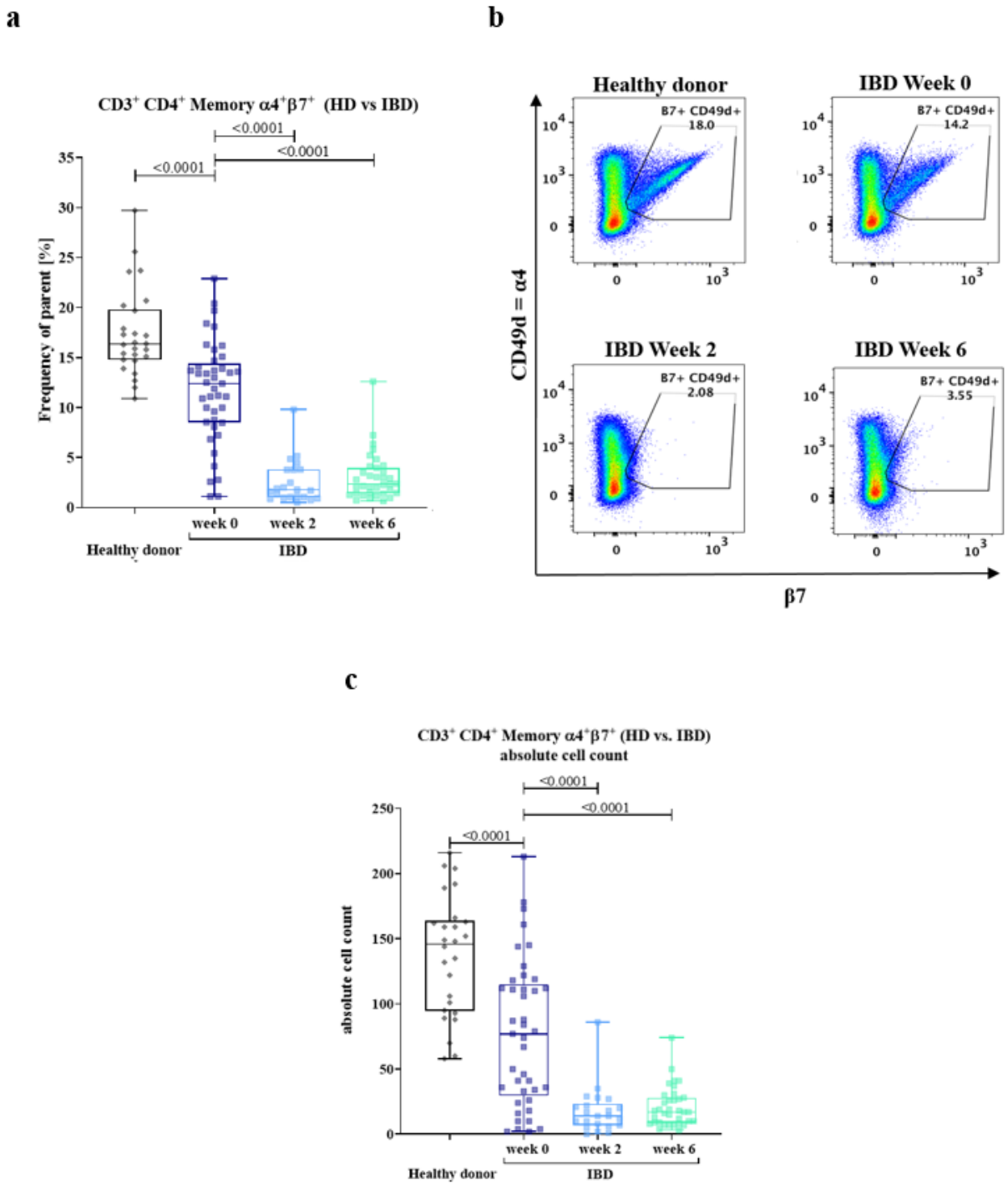


Figure 47: α4β7-expression on CD4+ memory T cells: Comparison of healthy donors and IBD patients

(gated on live cells -> single cells -> CD3+ -> CD4+ -> CD45RA-)
***a**: Comparison of healthy donors and IBD patients (frequency of parent); n (HD) = 26; IBD: n (week 0) = 41; n (week 2) = 22; n (week 6) = 35; test used for HD vs IBD week 0: Mann-Whitney Test; test used for IBD week 0 vs week 2 and week 6: Wilcoxon Test*
***b**: Examples of flow cytometry plots showing the differences between healthy donors and the different time points*
***c**: Comparison of healthy donors and IBD patients (absolute cell count); same cohort and tests used as in a*

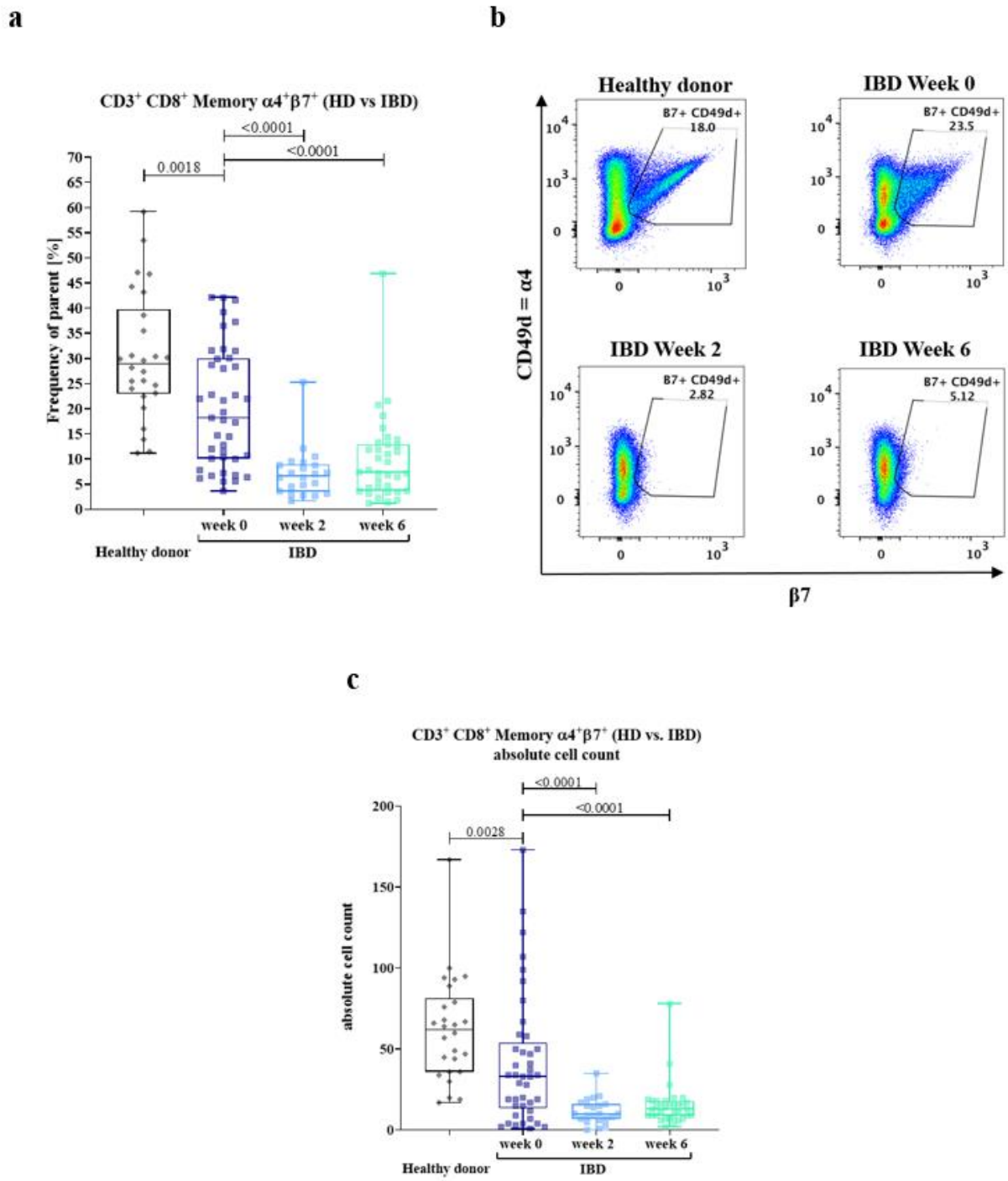


Figure 48: α4β7-expression on CD8+ memory T cells: Comparison of healthy donors and IBD patients

(gated on live cells -> single cells -> CD3+ -> CD8+ -> CD45RA-)
***a**: Comparison of healthy donors and IBD patients (frequency of parent); n (HD) = 26; IBD: n (week 0) = 41; n (week 2) = 22; n (week 6) = 35; test used for HD vs IBD week 0: Mann-Whitney Test; test used for IBD week 0 vs week 2 and week 6: Wilcoxon Test*
***b**: Examples of flow cytometry plots showing the differences between healthy donors and the different time points*
***c**: Comparison of healthy donors and IBD patients (absolute cell count); same cohort and tests used as in a*

The comparison of responders and non-responders in $CD4^+$ memory T cells showed a higher median of non-responders at baseline (1.1% vs. 13.7%; see Figure 49a). This turned in week 2 and 6. In $CD8^+$ memory T cells the median of non-responders was always higher through the induction phase (see Figure 50a). However, α4β7-Integrin could not statistically significantly predict therapy response during the first six weeks of the treatment.

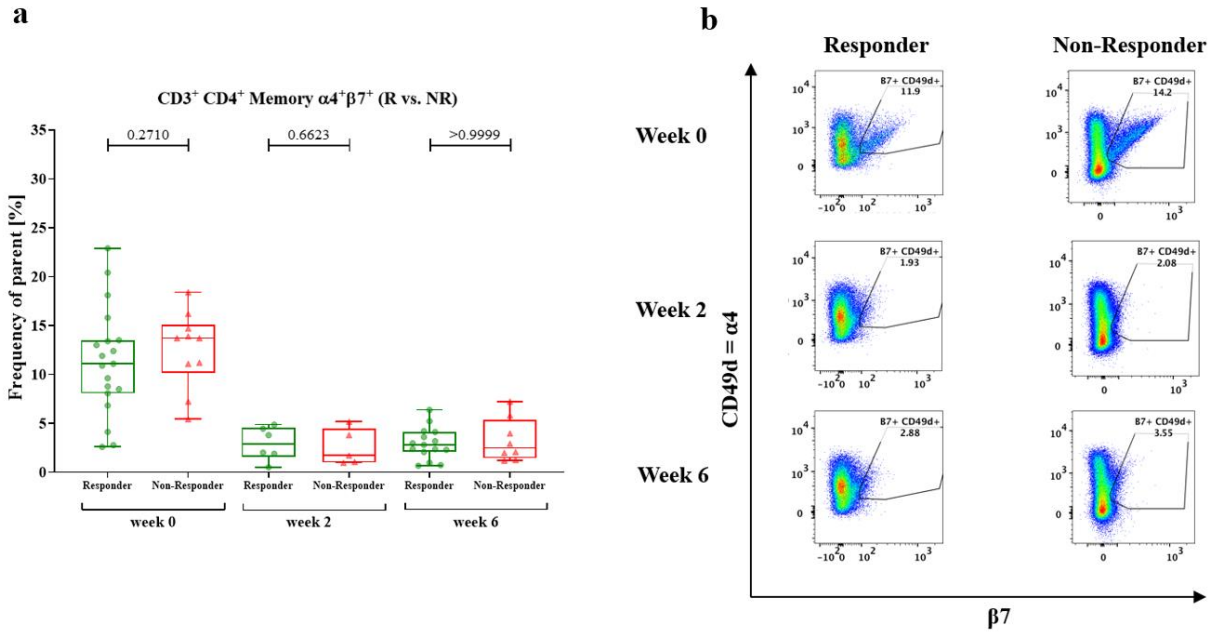


Figure 49: α4β7-expression on $CD4^+$ memory T cells: Comparison of responders and non-responders

(gated on live cells -> single cells -> $CD3^+$ -> $CD4^+$ -> $CD45RA^-$)
***a**: Comparison of α4β7-expression on $CD4^+$ memory T cells in responders and non-responders; responder: n (week 0) = 19; n (week 2) = 6; n (week 6) = 15; non-responder: n (week 0) = 10; n (week 2) = 5; n (week 6) = 8; test used: Mann-Whitney-Test*
***b**: Examples of flow cytometry plots showing the differences of α4β7-expression on $CD4^+$ memory T cells between responders and non-responders during the different weeks*

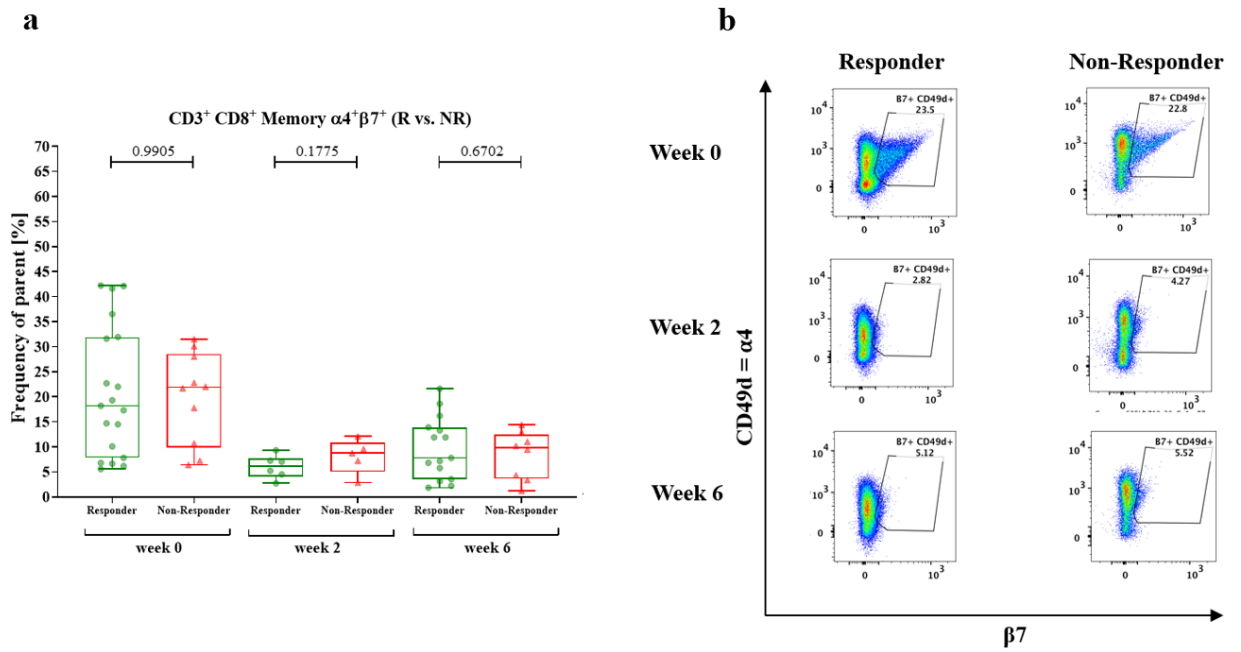


Figure 50: α4β7-expression on CD8$^+$ memory T cells: Comparison of responders and non-responders

(gated on live cells -> single cells -> CD3$^+$ -> CD8$^+$ -> CD45RA$^-$)
***a**: Comparison of α4β7-expression on CD8$^+$ memory T cells in responders and non-responders; responder: n (week 0) = 19; n (week 2) = 6; n (week 6) = 15; non-responder: n (week 0) = 10; n (week 2) = 5; n (week 6) = 8; test used: Mann-Whitney-Test*
***b**: Examples of flow cytometry plots showing the differences of α4β7-expression on CD4$^+$ memory T cells between responders and non-responders during the different weeks*

The other heterodimer combination of α4 - α4$^+$β1$^+$ - was significantly higher in relative numbers in IBD patients at baseline compared to healthy donors in CD4$^+$ memory T cells (45.95% vs. 38.4%; see Figure 51a). This could not be shown as in absolute numbers (see Figure 51c). During therapy the median level dropped to 39.95% in week 2 to recover to 43.95% in week 6. However, this comparison was statistically insignificant.

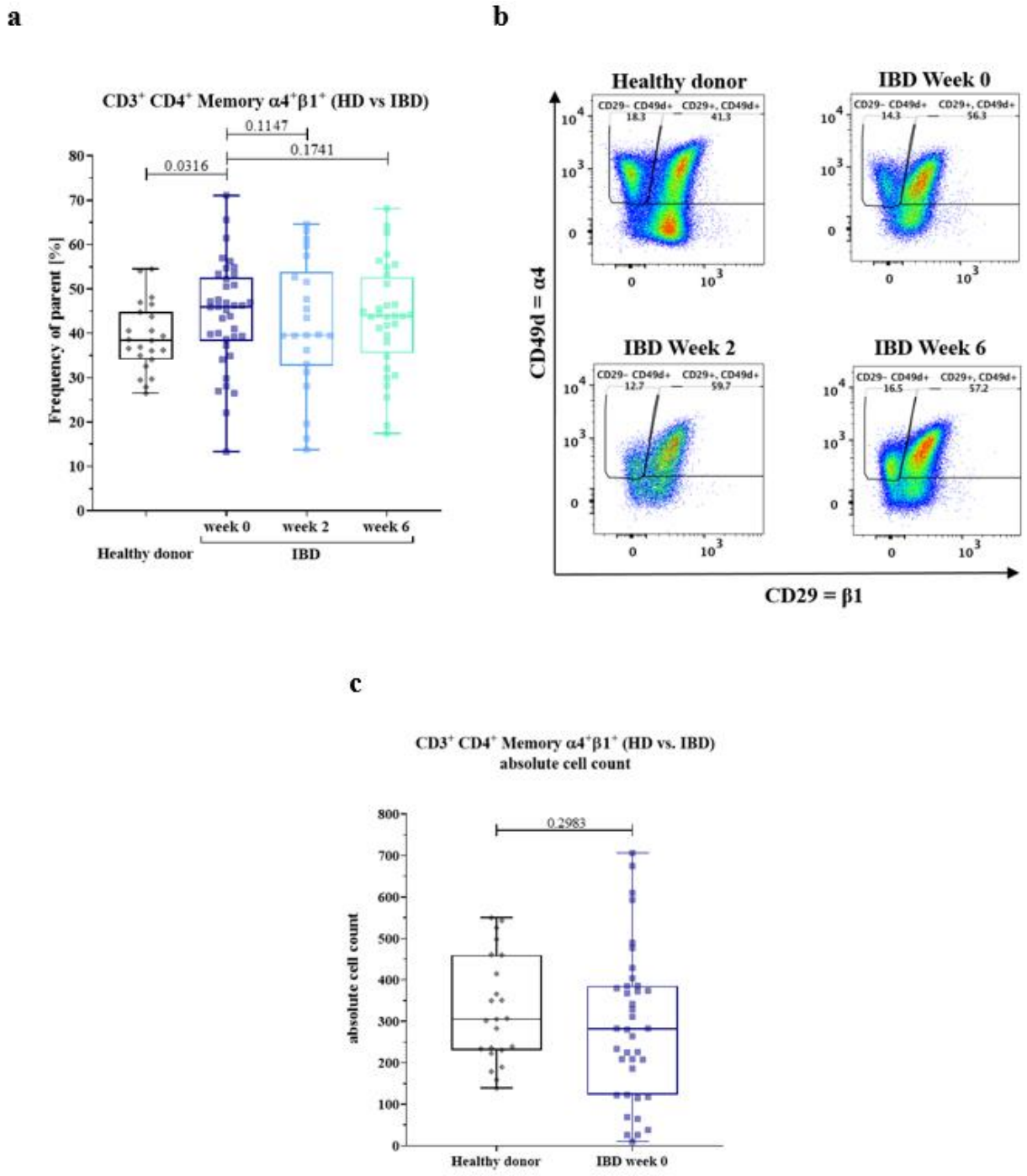


Figure 51: α4β1-expression on CD4+ memory T cells: Comparison of healthy donors and IBD patients

(gated on live cells -> single cells -> CD3+ -> CD4+ -> CD45RA-)
***a**: Comparison of healthy donors and IBD patients (frequency of parent); n (HD) = 23; IBD: n (week 0) = 48; n (week 2) = 22; n (week 6) = 32; test used for HD vs IBD week 0: Mann-Whitney Test; test used for IBD week 0 vs week 2 and week 6: Wilcoxon Test*
***b**: Examples of flow cytometry plots showing the differences between healthy donors and the different time points*
***c**: Comparison of healthy donors and IBD patients (absolute cell count); same cohort and tests used as in a*

Comparing α4β1-expression on $CD4^+$ memory T cells in responders and non-responders showed similar median levels in week 0 and week 6. Only at week 2 responders had a higher median level than non-responders (43.95% vs. 39.5%; see Figure 52a). However, at no timepoint a statistically significant prediction of therapy outcome could be made.

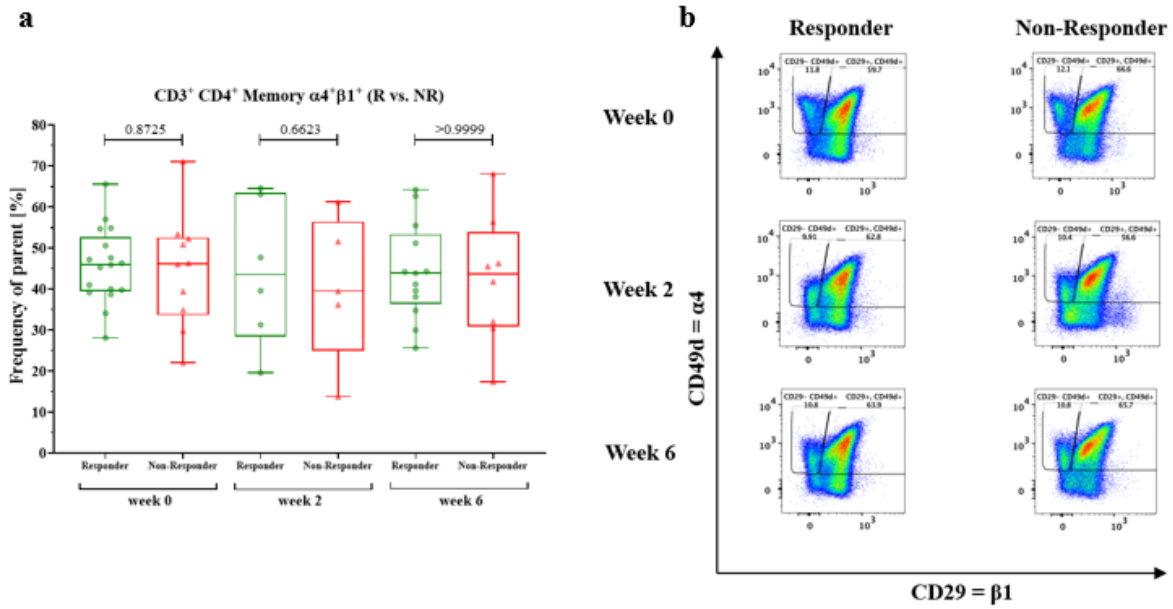


Figure 52: α4β1-expression on $CD4^+$ memory T cells: Comparison of responders and non-responders

(gated on live cells -> single cells -> $CD3^+$ -> $CD4^+$ -> $CD45RA^-$)
***a**: Comparison of responders and non-responders*; *responder: n (week 0) = 17; n (week 2) = 6; n (week 6) = 13; non-responder: n (week 0) = 10; n (week 2) = 5; n (week 6) = 8; test used: Mann-Whitney-Test*
***b**: Examples of flow cytometry plots showing the differences between responders and non-responders during the different weeks*

On $CD8^+$ memory T cells no statistically significant difference could be found between IBD patients at baseline and healthy donors (23.5% vs. 22.7%; see Figure 53a) nor during therapy (week 2: 23.35%; week 6: 26%). Comparing responders and non-responders showed similar median levels at baseline and week 6. Only at week 2 responders had a higher median level with 20.45% compared to 14.7% (see Figure 53c). However, this comparison statistically insignificant.

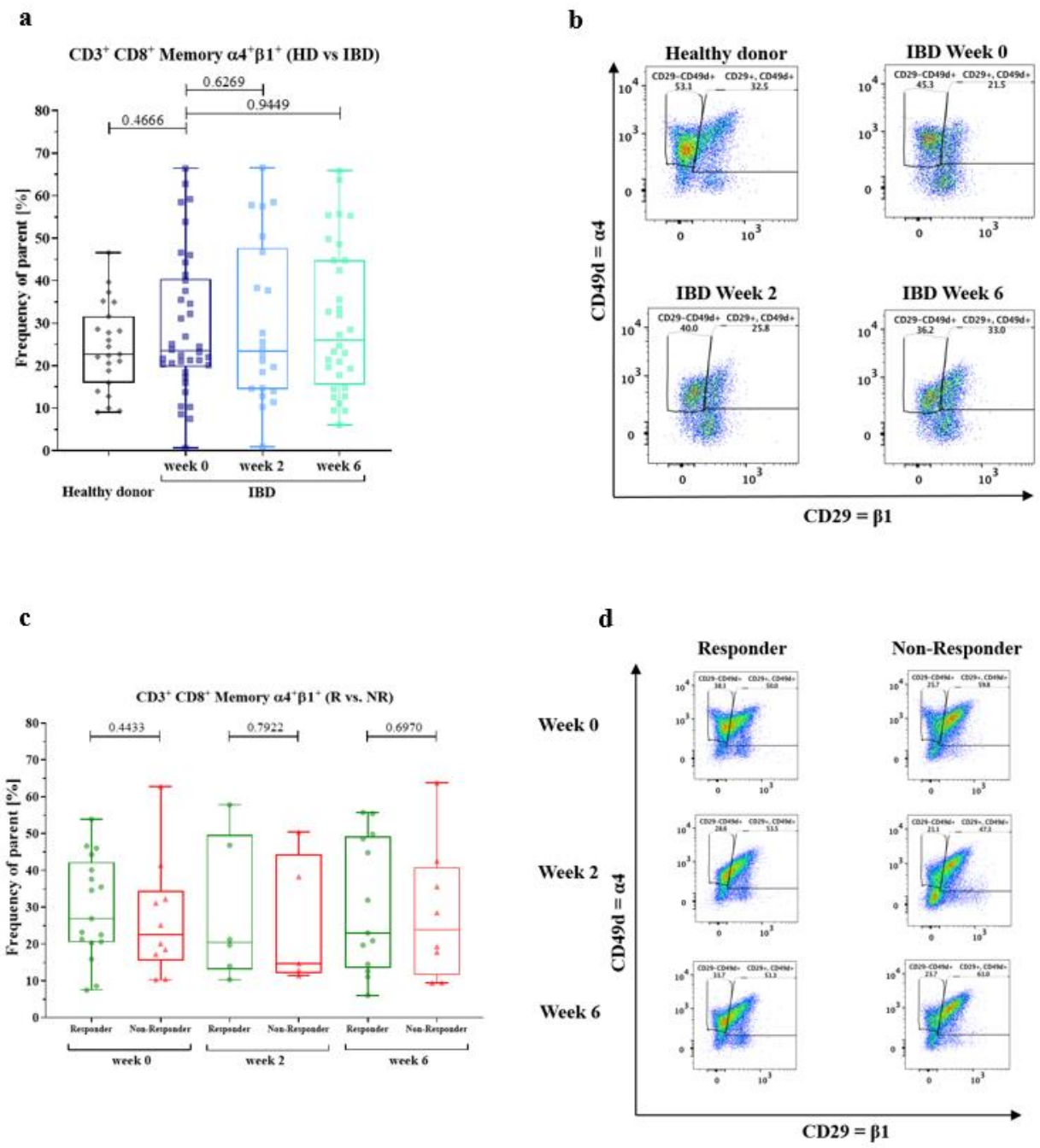


Figure 53: α4β1-expression on CD8+ memory T cells

(gated on live cells -> single cells -> CD3+ -> CD8+ -> CD45RA-)
***a**: Comparison of healthy donors and IBD patients (frequency of parent); n (HD) = 23; IBD: n (week 0) = 38; n (week 2) = 22; n (week 6) = 32; test used for HD vs IBD week 0: Mann-Whitney Test; test used for IBD week 0 vs week 2 and week 6: Wilcoxon Test*
***b**: Examples of flow cytometry plots showing the differences between healthy donors and the different time points*
***c**: Comparison of responders and non-responders; responder: n (week 0) = 17; n (week 2) = 6; n (week 6) = 13; non-responder: n (week 0) = 10; n (week 2) = 5; n (week 6) = 8; test used: Mann-Whitney-Test*
***d**: Examples of flow cytometry plots showing the differences between responders and non-responders during the different weeks*

The last possible integrin combination - αEβ7 – was statistically significantly lower in IBD patients at baseline compared to healthy donors in relative and absolute numbers in $CD4^+$ memory T cells (median percentage: 0.16% vs. 0.3%; see Figure 54a; median absolute numbers: 1 vs. 3; see Figure 55). The level statistically significantly decreased during therapy in relative numbers but not in absolute numbers. However, a similar trend could be seen with a median of 0.08% at week 2 and 0.08% at week 6.

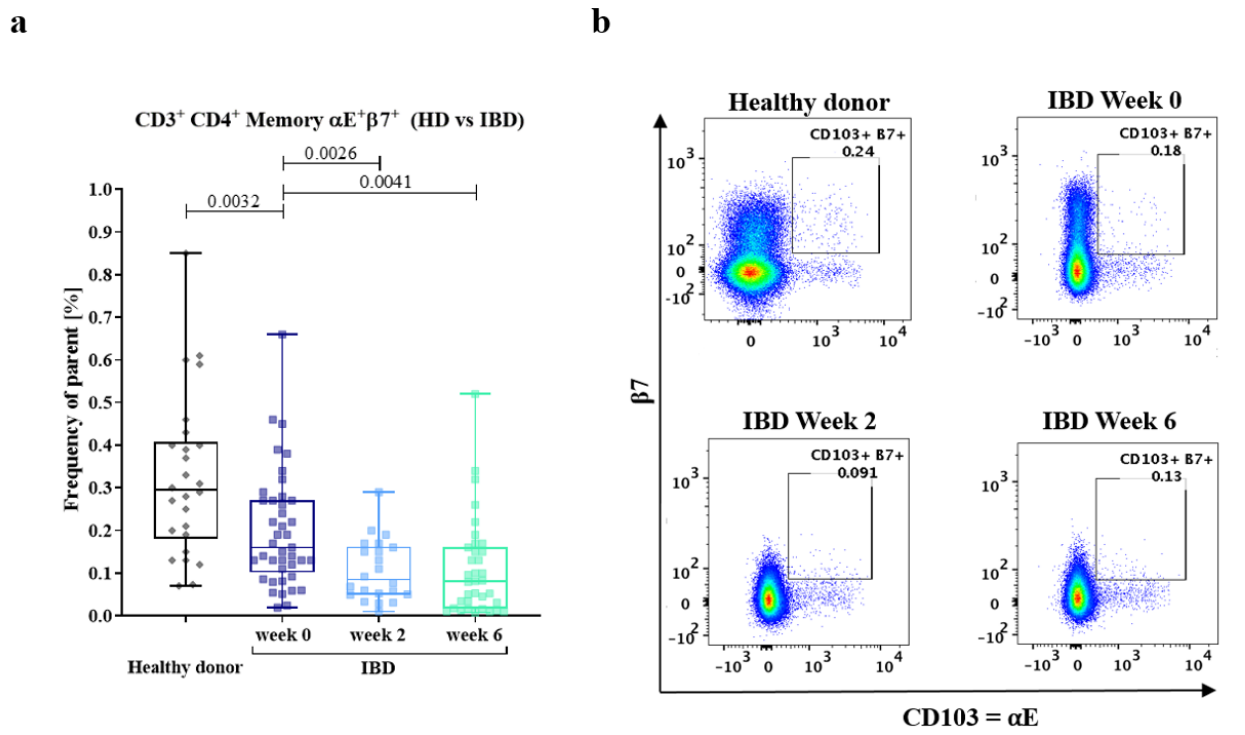


Figure 54: αEβ7-expression on $CD4^+$ memory T cells: Comparison of healthy donors and IBD patients

(gated on live cells -> single cells -> $CD3^+$ -> $CD4^+$ -> $CD45RA^-$)
***a**: Comparison of healthy donors and IBD patients (frequency of parent); n (HD) = 26; IBD: n (week 0) = 41; n (week 2) = 22; n (week 6) = 35; test used for HD vs IBD week 0: Mann-Whitney Test; test used for IBD week 0 vs week 2 and week 6: Wilcoxon Test*
***b**: Examples of flow cytometry plots showing the differences between healthy donors and the different time points*

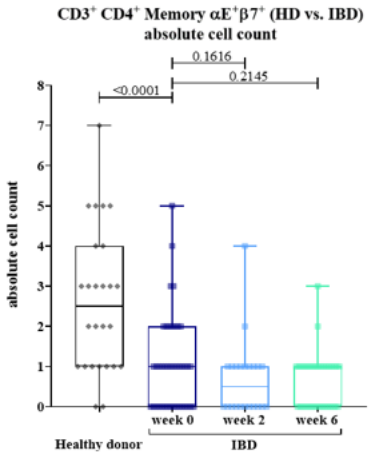


Figure 55: αEβ7-expression on CD4$^+$ memory T cells: Comparison of healthy donors and IBD patients (absolute cell count)

Comparison of healthy donors and IBD patients (absolute cell count); n (HD) = 26; IBD: n (week 0) = 41; n (week 2) = 22; n (week 6) = 35; test used for HD vs IBD week 0: Mann-Whitney Test; test used for IBD week 0 vs week 2 and week 6: Wilcoxon Test

In CD8$^+$ memory T cells the similar comparison could be made: IBD patients had statistically significant lower percentage and absolute numbers at baseline compared to healthy donors (median percentage: 1.99% vs. 4.52%; see Figure 56a; median absolute numbers: 3 vs. 8; see Figure 56c). The level then decreased during therapy. But this time the trend was statistically significant in both relative and absolute numbers (see Figure 56a and c).

In both CD4$^+$ and CD8$^+$ memory T cells the expression of αEβ7-Integrin could not predict response except at week 2 on CD8$^+$ memory T cells, when non-responders a higher median of 1.72% vs. 0.66% (see Figure 57). Nevertheless, this significant difference could not be shown at week 0 nor week 6.

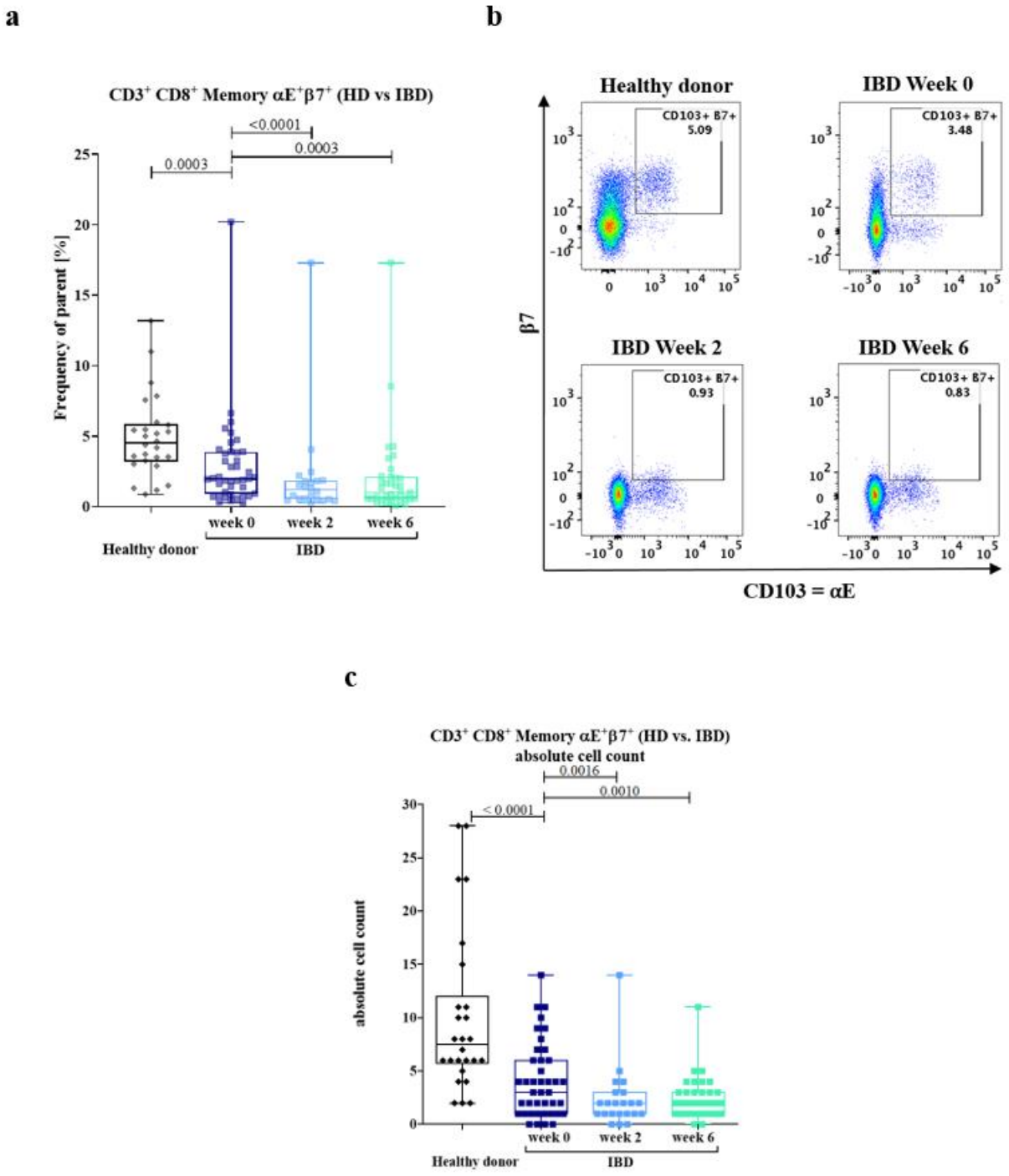


Figure 56: αEβ7-expression on CD8$^+$ memory T cells: Comparison of healthy donors and IBD patients

(gated on live cells -> single cells -> CD3$^+$ -> CD8$^+$ -> CD45RA$^-$)
***a**: Comparison of healthy donors and IBD patients (frequency of parent); n (HD) = 26; IBD: n (week 0) = 41; n (week 2) = 22; n (week 6) = 35; test used for HD vs IBD week 0: Mann-Whitney Test; test used for IBD week 0 vs week 2 and week 6: Wilcoxon Test*
***b**: Examples of flow cytometry plots showing the differences between healthy donors and the different time points*
***c**: Comparison of healthy donors and IBD patients (absolute cell count); same cohort and tests used as in a*

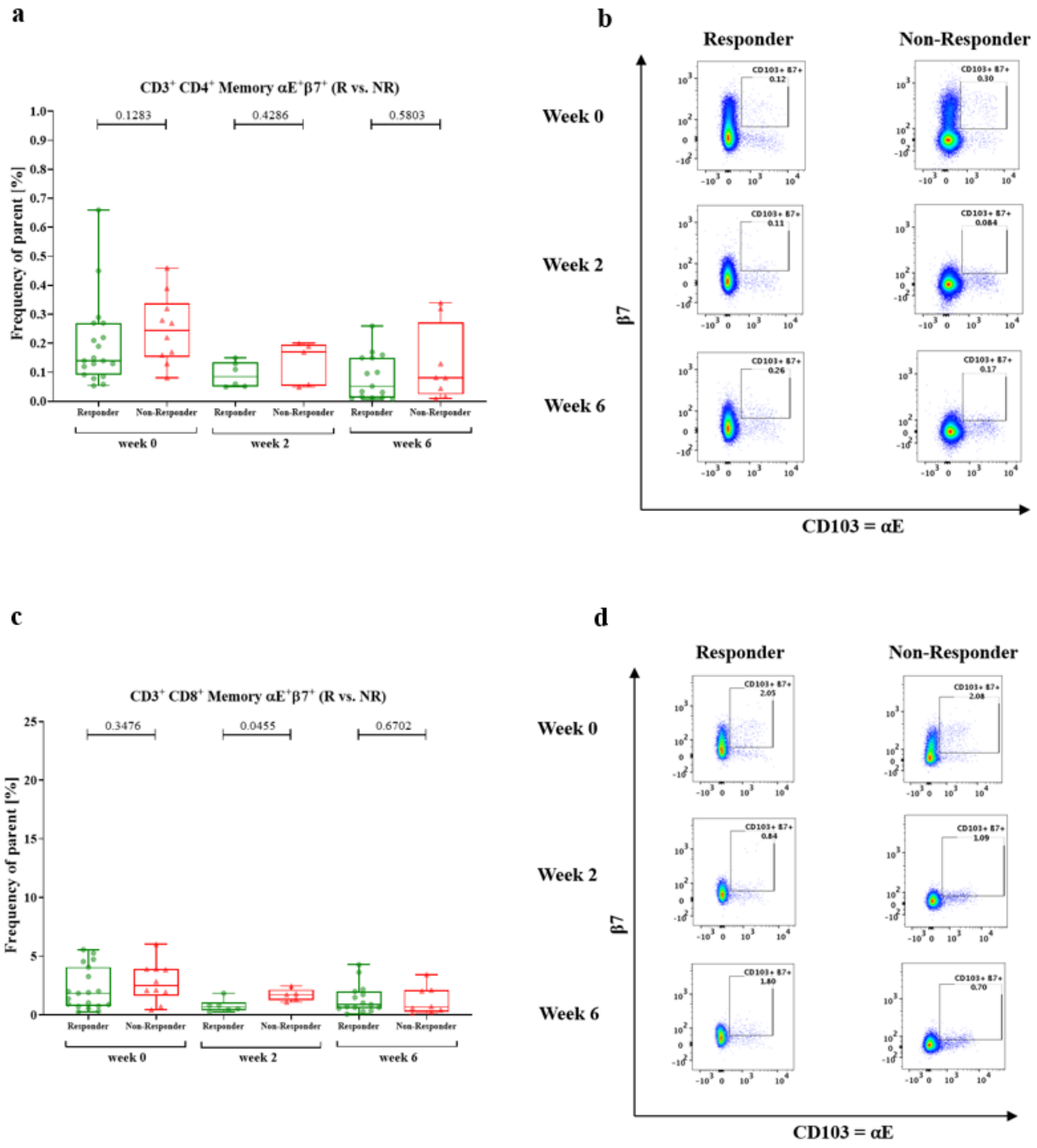
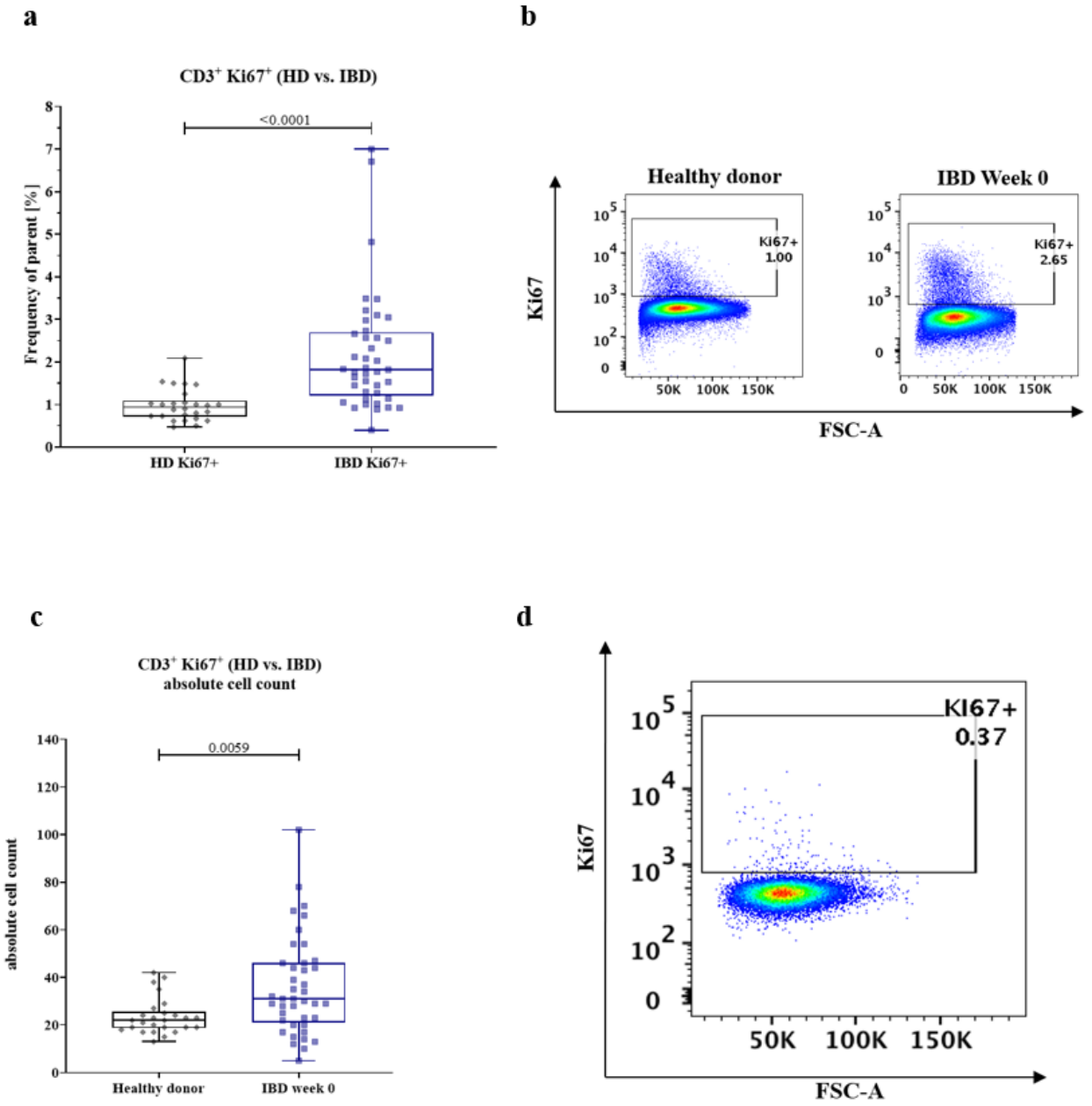


Figure 57: αEβ7-expression on $CD4^+$ and $CD8^+$ memory T cells: Comparison of responders and non-responders

(gated on live cells -> single cells -> $CD3^+$ -> $CD4^+$ and $CD8^+$ -> $CD45RA^-$)
***a**: Comparison of αEβ7-expression on $CD4^+$ memory T cells in responders and non-responders; responder: n (week 0) = 19; n (week 2) = 6; n (week 6) = 15; non-responder: n (week 0) = 10; n (week 2) = 5; n (week 6) = 8; test used: Mann-Whitney-Test*
***b**: Examples of flow cytometry plots showing the differences of αEβ7-expression on $CD4^+$ memory T cells between responders and non-responders during the different weeks*
***c**: Comparison of αEβ7-expression on $CD8^+$ memory T cells in responders and non-responders; same cohort and tests used as in a*
***d**: Examples of flow cytometry plots showing the differences of αEβ7-expression on $CD8^+$ memory T cells between responders and non-responders during the different weeks*

4.3.7 Ki67 expression

Several other markers were checked for their predictive power for therapy outcome. The subset which showed the highest power was Ki67$^+$ T lymphocytes. These are already statistically significantly increased in IBD patients before therapy compared to healthy donors in relative and absolute numbers (median percentage: 1.82% vs. 0.94%; median absolute numbers: 31 vs. 22; see Figure *58: Ki67-expression on T cells: Comparison of healthy donors and IBD patients*



(gated on live cells -> single cells -> CD3+)
***a**: Comparison of healthy donors and IBD patients at week 0 (frequency of parent); n (HD) = 26; IBD: n (week 0) = 41; test used for HD vs IBD week 0: Mann-Whitney Test*
***b**: Examples of flow cytometry plots showing the differences between healthy donors and IBD patients at week 0*

***c**: Comparison of healthy donors and IBD patients at week 0 (absolute cell count); same cohort and tests used as in a*
***d:** Control of Ki67-staining on naïve CD4+ T cells, which show no expression*Figure 58a and c).

Figure 58: Ki67-expression on T cells: Comparison of healthy donors and IBD patients

(gated on live cells -> single cells -> $CD3^+$)
***a**: Comparison of healthy donors and IBD patients at week 0 (frequency of parent); n (HD) = 26; IBD: n (week 0) = 41; test used for HD vs IBD week 0: Mann-Whitney Test*
***b**: Examples of flow cytometry plots showing the differences between healthy donors and IBD patients at week 0*
***c**: Comparison of healthy donors and IBD patients at week 0 (absolute cell count); same cohort and tests used as in a*
***d:** Control of Ki67-staining on naïve $CD4^+$ T cells, which show no expression*

Comparing its expression in responders and non-responders before even beginning the therapy demonstrated a significantly higher expression in non-responders compared to non-responders in percentage numbers (2.88% vs. 1.72%; see Figure 59a). The absolute cell numbers show the same trend (median: 36 vs. 28), even though the difference did not reach a significant level (see Figure 60).

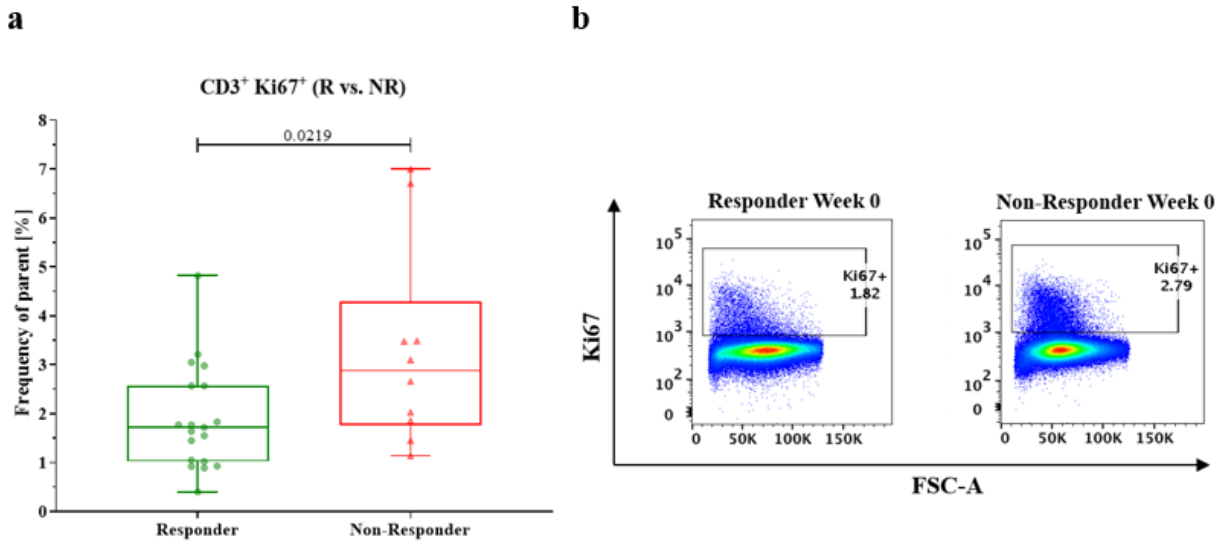


Figure 59: Ki67-expression on T cells: Comparison of responders and non-responders

(gated on live cells -> single cells -> $CD3^+$)
***a**: Comparison of responders and non-responders at week 0 (frequency of parent); n (responders) = 19; n (non-responders) = 10; test used for HD vs IBD week 0: Mann-Whitney Test*
***b**: Examples of flow cytometry plots showing the differences between responders and non-responders at week 0*

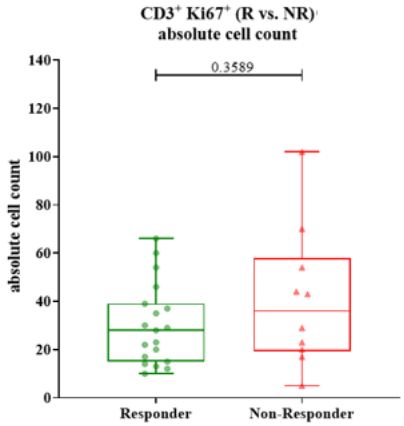


Figure 60: Ki67-expression on T cells: Comparison of responders and non-responders (absolute cell count)

(gated on live cells -> single cells -> CD3$^+$)
Comparison of responders and non-responders at week 0 (absolute cell count); n (responders) = 19; n (non-responders) = 10; test used for HD vs IBD week 0: Mann-Whitney Test

I further classified which T lymphocyte subgroups specifically express Ki67$^+$: It could be shown that in both responders and non-responders the CD4$^+$ T lymphocytes were statistically significantly more expressed in the Ki67$^+$ subgroup (see Figure 61). The significance could only be shown in percentage not in absolute numbers except for responders.

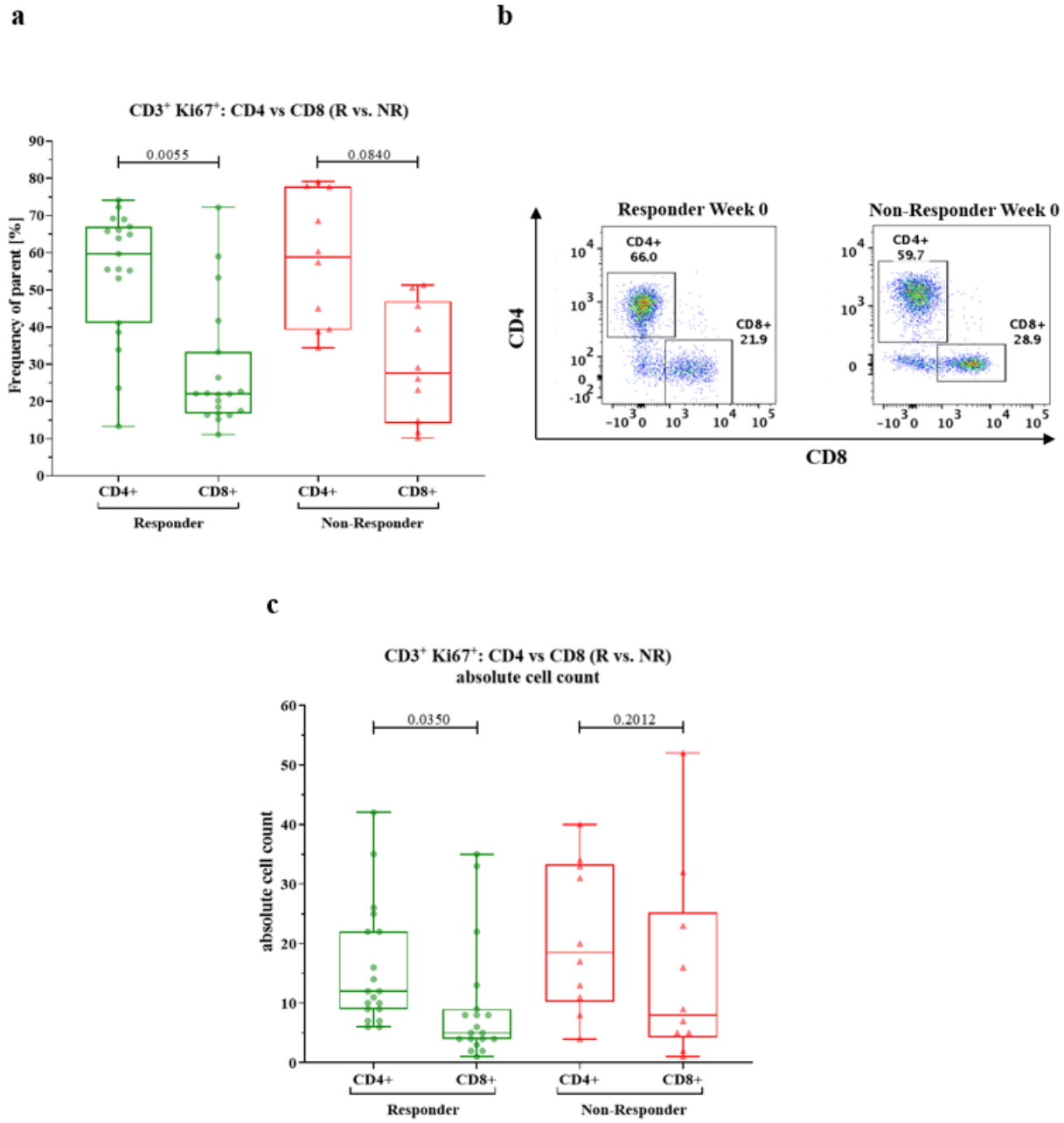


Figure 61: Comparison of CD4$^+$ and CD8$^+$ portion in Ki67$^+$ T cells: Comparison of responders and non-responders

(gated on live cells -> single cells -> CD3$^+$ -> Ki67$^+$)
***a**: Comparison of CD4$^+$ and CD8$^+$ percentage in Ki67$^+$ T cells in responders and non-responders at week 0 (frequency of parent); n (responders) = 19; n (non-responders) = 10; test used for HD vs IBD week 0: Mann-Whitney Test*
***b**: Examples of flow cytometry plots showing the differences between responders and non-responders at week 0*
***c**: Comparison of CD4$^+$ and CD8$^+$ percentage in Ki67$^+$ T cells in responders and non-responders at week 0 (absolute cell count); same cohort and tests used as in a*

When gating on $CD4^+$ most of the cells turned out to be – statistically significant in both relative and absolute numbers – memory T cells (see Figure 62).

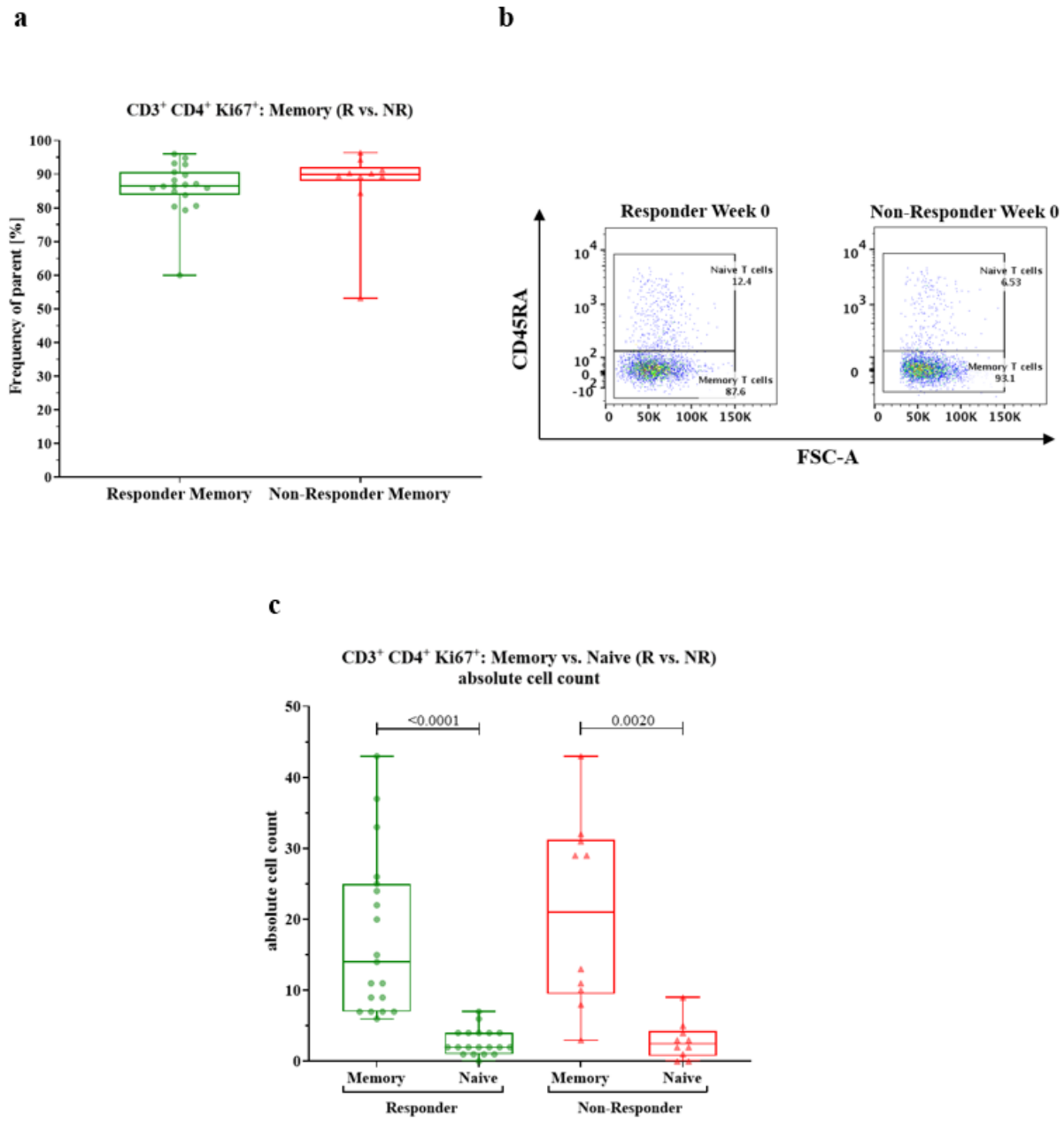


Figure 62: Comparison of memory and naïve T cell portion in $Ki67^+$ $CD4^+$ T cells: Comparison of responders and non-responders

(gated on live cells -> single cells -> $CD3^+$-> $CD4^+$ -> $Ki67^+$)
***a**: Comparison of memory and naïve T cell percentage in $Ki67^+$ $CD4^+$ T cells in responders and non-responders at week 0 (frequency of parent); for simpler structure only memory T cells shown; n (responders) = 19; n (non-responders) = 10; test used for HD vs IBD week 0: Mann-Whitney Test*
***b**: Examples of flow cytometry plots showing the differences between responders and non-responders at week 0*
***c**: Comparison of memory and naïve T cell percentage in $Ki67^+$ $CD4^+$ T cells in responders and non-responders at week 0 (absolute cell count); same cohort and tests used as in a*

Further subdividing the memory T cells showed that most of the Ki67$^+$ CD4$^+$ memory T cells were – statistically significant in both relative and absolute numbers – T_{EM} (see Figure 63).

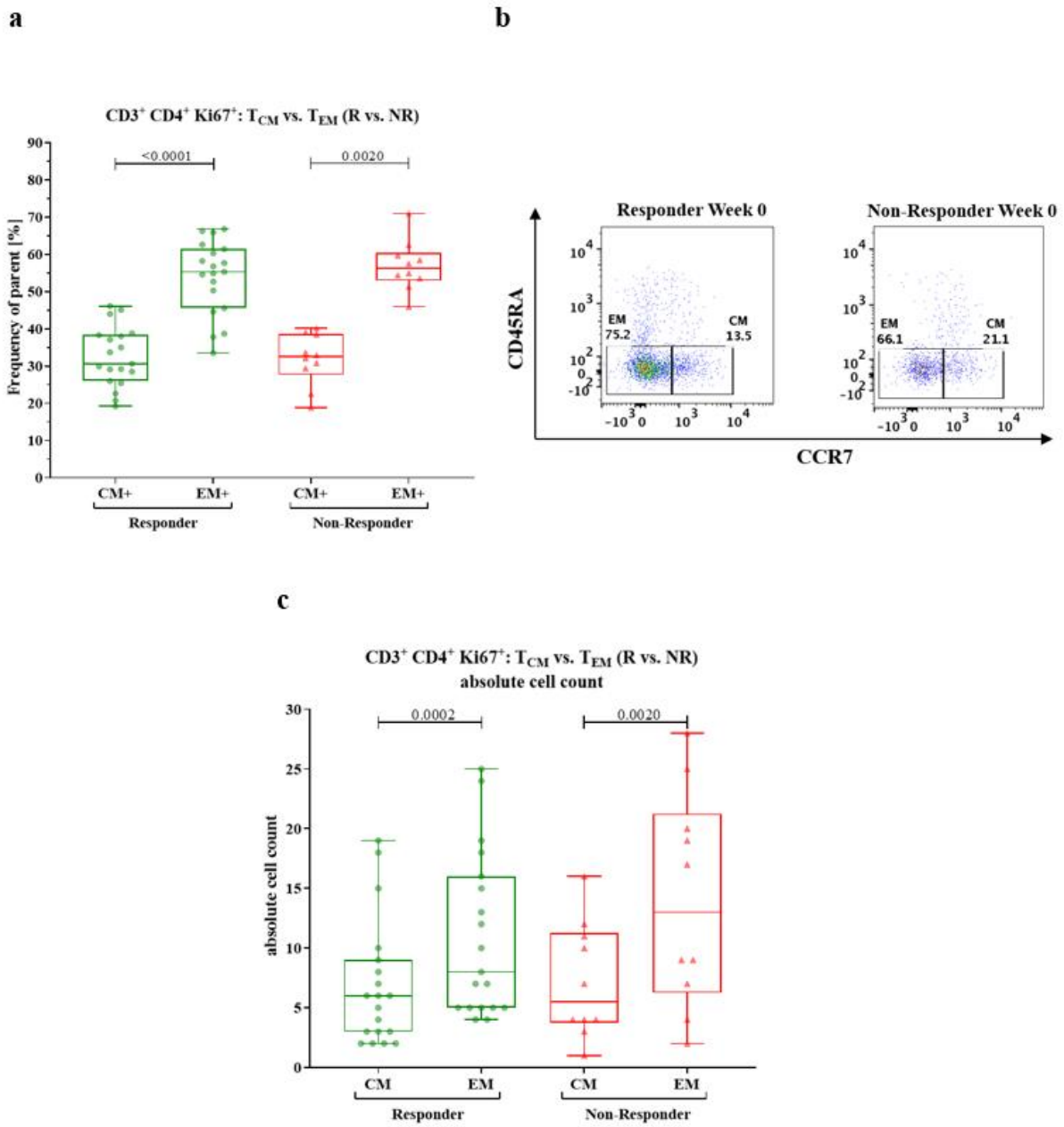


Figure 63: Comparison of central and effector memory portion in Ki67$^+$ CD4$^+$ T cells: Comparison of responders and non-responders

(gated on live cells -> single cells -> CD3$^+$ -> CD4$^+$ -> Ki67$^+$)
***a**: Comparison of the percentage of central and effector memory T cells in Ki67$^+$ CD4$^+$ T cells in responders and non-responders at week 0 (frequency of parent); n (responders) = 19; n (non-responders) = 10; test used for HD vs IBD week 0: Mann-Whitney Test*
***b**: Examples of flow cytometry plots showing the differences between responders and non-responders at week 0*
***c**: Comparison of the percentage of central and effector memory T cells in Ki67$^+$ CD4$^+$ T cells in responders and non-responders at week 0 (absolute cell count); same cohort and tests used as in a*

I then analysed the expression of several surface markers on this subgroup ($CD4^+$ effector memory T cells which express $Ki67^+$): $CD38^+$ turned out to be statistically significantly higher expressed in non-responders than responders in percentage but not in absolute numbers (see Figure 64).

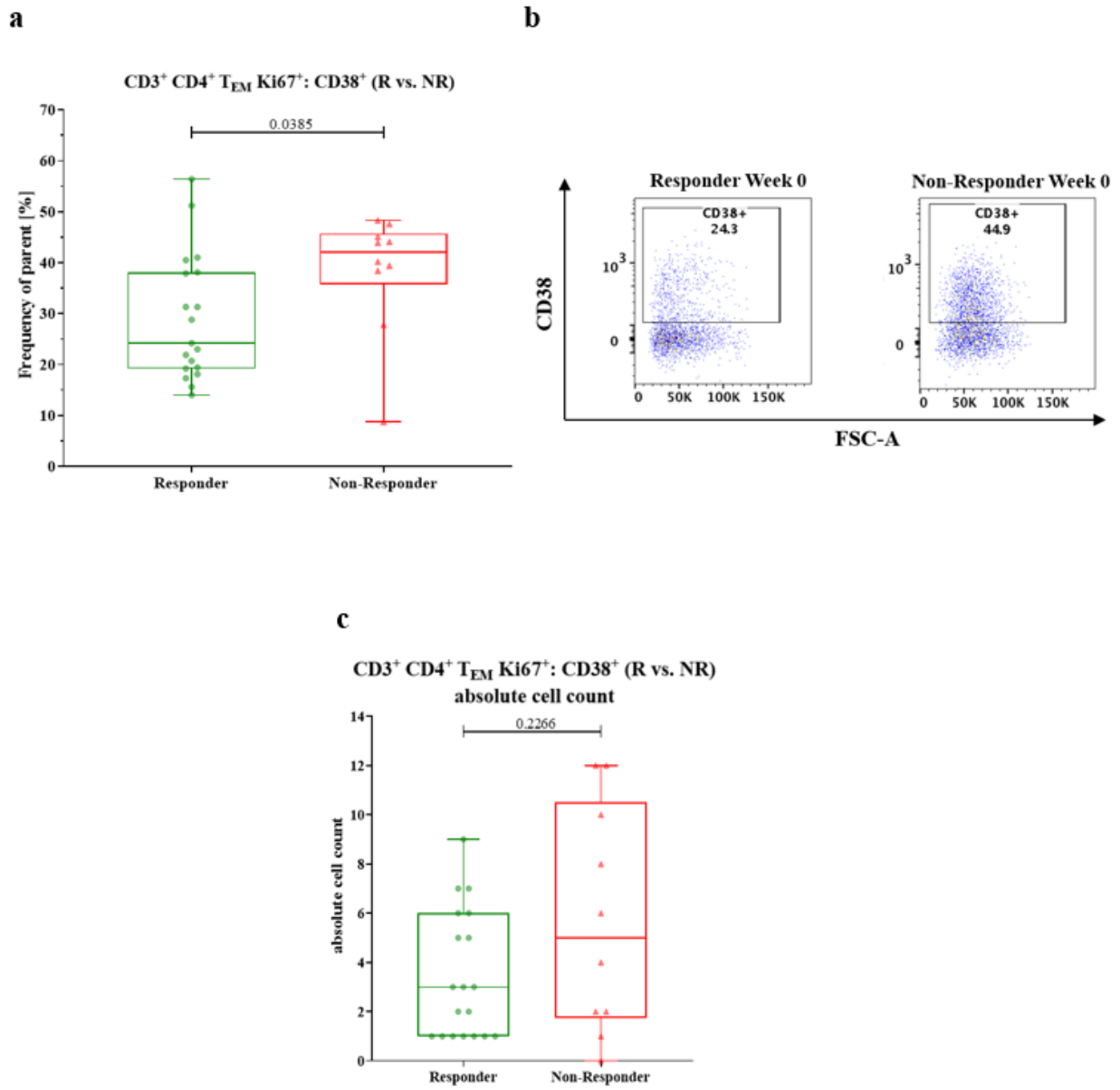


Figure 64: CD38-expression on $Ki67^+$ $CD4^+$ central memory T cells: Comparison of responders and non-responders

(gated on live cells -> single cells -> $CD3^+$ -> $CD4^+$ -> $CD45RA^-$ $CCR7^-$ -> $Ki67^+$)
***a**: Comparison of the percentage of CD38-expression on $Ki67^+$ $CD4^+$ effector memory T cells in responders and non-responders at week 0 (frequency of parent); n (responders) = 19; n (non-responders) = 10; test used for HD vs IBD week 0: Mann-Whitney Test*
***b**: Examples of flow cytometry plots showing the differences between responders and non-responders at week 0*
***c**: Comparison of the percentage of CD38-expression on $Ki67^+$ $CD4^+$ effector memory T cells in responders and non-responders at week 0 (absolute cell count); same cohort and tests used as in a*

Thus, the Ki67$^+$ T lymphocytes and its subgroup CD4$^+$ effector memory T cells which express Ki67$^+$ and CD38 showed good predictive power in terms of therapy response already at week 0. So I built a ROC-Curve for both populations (see Figure 65): It showed that a frequency of more than 1.845% of Ki67$^+$ on T lymphocytes predicted with an 80% sensitivity and 68.42% specificity, that the patient would not respond to the vedolizumab therapy. As I gated on CD4$^+$ effector memory T cells, which are Ki67$^+$ and CD38$^+$, I could predict non-response with the same sensitivity of 80% but a higher specificity of 78.95% if the frequency CD38 would be more than 38.25%.

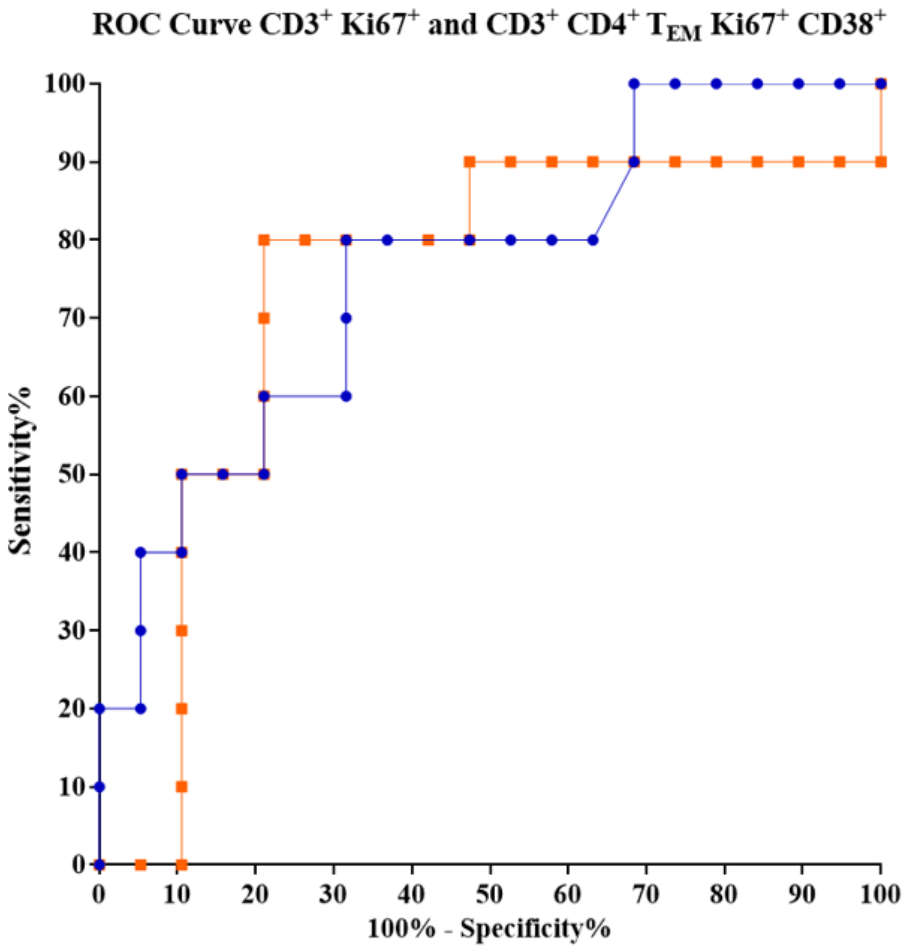


Figure 65: Comparison of ROC-Curves of Ki67-expression on T cells and CD38-expression on Ki67$^+$ CD4$^+$ central memory T cells

Blue: Ki67-expression on T cells, area under the curve: 0.7605
Orange: CD38-expression on Ki67$^+$ CD4$^+$ central memory T cells; area under the curve: 0.7368

5. Discussion

5.1 Clinical markers – predictive power

In summary, the clinical markers I analysed could not predict clinical response before beginning the vedolizumab treatment. Likewise, I could not find any significant differences between responders and non-responders during therapy except for five markers: CrP, calprotectin as well as MCV, MCH and thrombocyte count.

CrP and calprotectin were both statistically significantly higher at week 6 in non-responders compared to responders. This can be explained as both markers are linked to the inflammation process in the gut: CrP is an acute phase protein produced in the liver as a response to inflammation processes. Its level rises quickly and decreases "just as rapidly with the resolution of the condition" (100). That is why it can be viewed as an unspecific signal of inflammation in the body. Calprotectin is a S-100-Protein, which makes up about 60% of cytosolic proteins in neutrophils (101). Measuring its level in the stool correlates with the number of leukocytes in the gut tissue. Both markers - CrP and calprotectin - are general inflammation markers, which explains why they are higher in non-responders than in responders: The inflammation level did not decrease as much in non-responders as seen in responders through vedolizumab treatment. These markers are already used in clinical practice to monitor the inflammation level. Their value could be validated in this study. However, they only statistically significantly differentiate between responders and non-responders after six weeks. This indicates that they are suitable markers for the course of therapy but cannot predict its outcome beforehand.

MCV and MCH are both haematological markers used to define red blood cells: MCV describes the volume of each cell on average and MCH the average amount of haemoglobin each cell carries. In this study, it could be shown that MCV and MCH are statistically significantly lower in non-responders at week 6. Two arguments could explain these results: Many patients with chronic inflammation experience at some point a so-called anaemia of chronic disease. Its cause is not fully understood yet, but it is proposed that the immune activation with an increase in inflammatory cytokines increases hepcidin as well as decreases erythropoietin (102). This can lead to an iron deficiency in red blood cell production, which results in a microcytic, hypochromic anaemia. Such anaemia may show itself in low levels of MCV and MCH. Another possible reason which could add to the anaemia is gastrointestinal blood loss through bleeding, as it can occur in inflamed IBD sites in the gut. Haematological markers have already been discussed to have predictive power for

the therapy outcome of vedolizumab treatment. But studies so far discussed low haemoglobin to be predictive of therapy outcome (81,82,103). This would also fit into the picture of the anaemia of chronic disease. However, in this study, neither haemoglobin level nor erythrocyte count proved to be significantly different between responders and non-responders at any point in time.

The last clinical marker to be significant was the thrombocyte count at week 6: The level was significantly higher in non-responders compared to responders. This can be explained by an interaction between inflammation processes and the thrombocyte function, which has been described in many studies. Thrombocytes themselves play an active role in inflammation. They release "platelet factor 4 (PF4), beta-thromboglobulin (bTG), platelet-derived growth factor (PDGF) and histamine-releasing factor (HRF) which are potent amplifiers of basophil, mast cell and neutrophil activity" (104). But high inflammation activity can also influence prothrombotic pathways as it activates the coagulation cascade and inhibits anticoagulant mechanisms – for example, protein C (105). Furthermore, inflammation markers can induce thrombopoiesis: Yan et al. showed that IBD is associated with a higher production of immature, hyperactive thrombocytes (105). These findings could also be why IBD patients generally have a higher risk of thromboembolism when compared with the healthy population (106). These studies underscore the value of my findings regarding the thrombocyte level.

In conclusion, all these markers are linked to a highly activated inflammation process, which can explain why they appear so strongly in non-responders as vedolizumab could not lower their disease activity. Nonetheless, these five clinical markers were only statistically significantly predictive at week 6, not at baseline. That is why they can be used as good monitoring markers but not as prediction markers.

5.2 Trough level – predictive power

My other attempt to find a predictive marker was measuring the trough level of vedolizumab at week 6. Even though the trough level did not show a significant difference between responders and non-responders, a trend could be seen with the average level in responders being higher (30.73 µg/ml vs. 21.88 µg/ml). These findings are in line with other studies: Dreesen et al. used a cut-off of 24.0 µg/ml as a level to differentiate responders from non-responders (82) and Hanzel et al. chose 22 µg/ml (83). The cohorts used in these previous studies are also comparable to the cohort used in this thesis, as all three have a high proportion of anti-TNF-treated patients. However, different outcome definitions of response could explain the differences in the cut-off levels.

These studies show that a larger cohort can statistically significantly predict therapy outcomes using the trough level of vedolizumab. This idea could lead to the suggestion that a higher vedolizumab dosage provides a better therapy outcome. To answer this question, an optimal dosage of vedolizumab must be found. At the moment, every patient – regardless of their weight – receives 300 mg vedolizumab, even though the post hoc analysis of the clinical trial studies already revealed that higher trough levels during induction correlated with a higher clinical remission rate (80). At a trough level of less than 17 µg/ml in UC and 16 µg/ml in CD patients´ clinical remission rates could not even be differentiated from the placebo group (80). But the European Medicines Agency (EMA) decided to allow vedolizumab as a fixed dose of 300mg. It argued that with a higher dose, the rate of anti-vedolizumab-antibodies would increase (107). The cut-off level for that would be 4 mg/kg. The EMA then used a 75 kg person as an average to argue 300mg as the standard dosage (107). An adjustment towards a weight-based system (4mg/kg * individual body weight of the patient) should be studied further.

However, just achieving higher trough levels does not seem to achieve clinical response so easily. Becker et al. proposed "a non-linear exposure-efficacy [of vedolizumab]" (108): They applied 10 µg/ml and 50 µg/ml (the most clinically relevant concentrations according to the authors) of fluorescently labelled vedolizumab to human T cells from patients with IBD in vitro. The authors could show that at 10 µg/ml more T effector cells (T_{EM}) were blocked by vedolizumab in comparison to regulatory T cells (T_{regs}) (108). At 50 µg/ml, both T cell subgroups were blocked equally (108). This data is important as T_{regs} are viewed to have a rather anti-inflammatory role compared to T_{eff} (109). Whether an ideal therapeutic window for vedolizumab treatment exists (108), as proposed by the authors, must be studied and validated in further clinical studies.

5.3 Flow cytometry data – Changes in T cell subsets

I could also show how vedolizumab influences different T cell subgroups and how this IBD cohort compares to healthy controls. The IBD patients had statistically significant fewer $CD3^+$ T lymphocytes in comparison to the healthy donor group in their blood. A possible explanation for this is that most of the $CD3^+$ T lymphocytes causing the inflammation are probably in the gut tissue and not in the bloodstream. The different subtypes of T lymphocytes did not show any significant differences, except for $CD8^+$ T_{EMRA}: IBD patients had a higher percentage number at baseline than healthy donors. This difference was non-significant in the absolute cell count, even though a trend could be seen.

A larger patient cohort could possibly show a significant difference. As T_{EMRA}-cells are often home to "sites of inflammation […] and display an immediate effector function [there]" (110), this hint could be explained.

In memory T cells in both $CD4^+$ and $CD8^+$ T cells I could show a significantly higher expression of CCR9 and GPR15 in IBD patients at baseline compared to healthy donors. Both are gut-homing markers on T and B cells: CCR9 mediates the entrance to the small intestine and GPR15 to the colon (111,112). This high expression can be explained as the influx of leukocytes to the gut is increased in IBD. However, both cannot predict the therapy outcome under vedolizumab treatment. Their expression is also not influenced by the antibody treatment.

The integrin heterodimer α4β1 could also not predict therapy outcome nor did vedolizumab influence its expression. This result leads to the conclusion that α4β1-integrin can still be used by T cells to enter the gut and bypass the vedolizumab blockade. However, Veny et al. could show that $\alpha4^+\beta1^+$ T cells (both $CD4^+$ and $CD8^+$) decrease in the gut tissue under vedolizumab therapy (113). The time span they monitored was much longer (until week 46). This might lead to the idea that the effect of vedolizumab on α4β1-integrin-expressing cells takes more time.

Responders and non-responders could also not be differentiated by αEβ7-expression. But IBD patients had statistically significant fewer $\alpha E^+\beta7^+$ -memory T cells in the blood. Its numbers decreased due to the therapy. The low numbers in general can be linked to the role of αEβ7 in retaining T cells in epithelial tissue by binding to E-cadherin (114,115). This would mean that most $\alpha E^+\beta7^+$ cells are already in the gut. The influence of vedolizumab on the remaining cells in the bloodstream could be explained by its interaction with the β7-integrin in the αEβ7-heterodimer.

I also investigated the expression of α4β7-integrin: I was able to measure α4β7-integrin after incubating them with vedolizumab for 30 minutes. Unfortunately, as I looked at the isolated cells from the study cohort, the integrin heterodimer could no longer be measured anymore after the beginning of the therapy. This finding has already been established, as the heterodimer gets internalized after vedolizumab binds to it. This has been proven in a preclinical in-vitro study by Wyant et al.: They incubated $\alpha4^+\beta7^+$ -cells at 37°C with fluorescently labelled vedolizumab for 24 hours and then examined them using microscopy (65): An internalisation into endosomes was monitored (65). That is why I conclude that the internalisation process needs between 30 minutes (time point I still could detect $\alpha4^+\beta7^+$ expression, while unmarked Vedolizumab was binding to it) and 24 hours (time point in the study by Wyant et al.).

α4β7-integrin could also not show any predictive power for therapy response in this study. A trend of higher expression in non-responders on both $CD4^+$ and $CD8^+$ memory T cells could be seen: A

significant trend has been detected by Schneider et al, as they gated on $CD3^+$ and $CD4^+$ T cells (89). Even though our cohorts are partly the same – as both our clinics work together in the IBDome study -, the analysis and interpretation were independently. Their possible explanation was that the expression of α4β7-integrin under the influence of Vedolizumab "inversely correlates with the capacity of T cells to adhere to MAdCAM-1" (89), as they detected in earlier studies (116 as cited in 89). Therefore, a huge part of α4β7-Integrin expressing cells in responders would be already in the gut and not in the bloodstream before therapy. Further investigation into this and into the mechanism of how α4β7-integrin is internalised by vedolizumab is needed. Another idea could just be that the higher amount of $\alpha4^+\beta7^+$ cells in non-responders overreaches the blocking capacity of vedolizumab leading to some cells not being blocked and being able to enter the gut.

5.4 Ki67-expression as a possible prediction marker

The marker with the most predictive power I found was $Ki67^+$. Its expression on lymphocytes was statistically significantly higher in non-responders than in responders. Looking at the different subgroups, Ki67 was predominantly expressed on $CD4^+$ effector memory T cells.

So far, Ki67 is mainly used as a marker in cancer diagnosis to see how fast a tumour is growing (117,118). Its functions have not been fully understood yet. Its role in regulating heterochromatin compaction and gene expression in proliferating cells could be shown (119). Furthermore, it helps to organize chromosome architecture and nucleolar during mitotic exit (120,121). Another discussed function is to support the removal of cytoplasm from the reassembling nucleus during mitotic exit (122). All these functions underline the important role of Ki67 in the cell cycle. Nevertheless, Sobecki et al. could show that Ki67-deficient mice are still vital and fertile (119). Its standing in the IBD pathogenesis has not been described yet. Its increased expression in non-responders shows that these patients have a higher rate of rapidly dividing cells, which increase the state of inflammation. This is supported by the fact that effector memory T cells, which are essential for keeping the autoinflammatory process going, have a high Ki67 expression.

The other marker which is statistically significantly increased on these cells is CD38: It has a "role in cell adhesion, migration and signal transduction" (123). This paints a picture of quickly multiplying cells, which can easily migrate into the gut to cause inflammation there. With these markers, a good prediction of therapy outcome was possible in my cohort.

The area under the curve, I could reach within the ROC curve for both $Ki67^+$ T lymphocytes and effector memory $CD4^+$ T cells, which express $Ki67^+$ and $CD38^+$, is also fairly satisfying considering the size of the patient cohort.

5.5 Limitations

However, the difference between responders and non-responders for Ki67-expression was only significant in percentage numbers, not in absolute numbers. A more extensive study population would be needed to see if the trend would turn out to be significant in absolute numbers. In general, the study population was too small for all the examined markers. A larger population could increase the statistical power of the ROC-curve. For its analysis the patients´ data was not split into a test train split, as the cohort size was too small for this. After all, this study was intended to be exploratory. Further clinical studies must follow.

As the cohort was so small, a differentiation between the single effect of vedolizumab and its comedication could not be made. Some responders may have profited more from their prednisolone dose and not directly responded to vedolizumab treatment. Another disadvantage of the study was that the collection of clinical markers was not standardised resulting in a heterogenous patient number in all analysed clinical markers. This can be explained as the decision on their assessment was made by the treating physician based on their acute use for further treatment steps.

In addition, I could not further subdivide the influence of vedolizumab on CD and UC. That would be especially important as many studies – even the clinical trial studies – could find a weaker effect of vedolizumab in CD patients (68,103). This hints to the discussed idea that CD could be stronger linked to a dysfunction in the innate rather than the adaptive immune system (124 as cited in 37).

Cell groups like Th1, Th2 and Th17 cells should also be analysed, which were spared out in this analysis. Furthermore, an intracellular staining of markers would not only be helpful to better understand the internalisation of α4β7-integrin but also give the possibility to check intracellular markers like transcription factors for their predictive power. To really complete the understanding of the different detected findings, gut samples would also be needed. This study focused on blood markers which could easily be measured in the clinical routine without a colonoscopy.

As I analysed different markers in the same cohort a multiple comparisons problem must be considered as well.

5.6 Outlook

Despite the shortcomings of this study, the results indicate tentatively that Ki67-expression could become a clinical decision tool upon IBD treatment and thereby improve patients' life. Besides replicating this study with a larger sample size and samples from the gut, future studies should investigate also whether the Ki67-expression is specific for non-response only to vedolizumab. It could be that a group of patients exists who are just difficult to treat with any biological drug. This investigation would be complicated by the fact that even within one patient, the inflammatory pathway can switch during the course of the disease (125) leading to a quite heterogeneous picture. Also, the group of remitters – patients who do not clearly fulfil the criteria for Response or Non-Response – has yet to be investigated further, as to how they can be better classified into the group of responders and non-responders or are a category of their own.

In the meantime, vedolizumab treatment could be improved by further analysing the combination potential of vedolizumab and other IBD medications. It has already been discussed to use thiopurines as a comedication of vedolizumab. The presumed advantage lies in the lower immunogenicity of vedolizumab (126). With that, higher trough levels could be achieved without fearing the production of anti-vedolizumab-antibodies. This could lead to higher response rates.

Another argument in favour of a combination therapy is that vedolizumab only blocks further immune cells from entering the gut. A drug with an instant effect in the inflamed tissue is needed for that.

In addition, discussions on how to improve the targeting of vedolizumab are needed: It only blocks α4β7-integrin but does not affect αEβ7-integrin nor α4β1-integrin. These were the lesson of natalizumab which targets α4-integrin in both its combinations effectively but was accompanied by a higher risk of PML. A promising idea was the development of etrolizumab, an anti-β7-antibody. However, its effectiveness is still under evaluation (127,128).

All this shows again that an effective marker for therapy prediction is needed. This study found that Ki67 could very well be such a marker. To validate this promising hypothesis, the main step is to initiate larger clinical studies. Ki67 could be integrated into a prediction model of more markers to increase accuracy. The need for such tools is not only highlighted by the economic cost of biological treatment but also the toll of various side effects and therapy failure on the patients' well-being.

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7. Supplemental

7.1 Eidesstattliche Versicherung

„Ich, Konstantin Fritz, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: „The impact of anti-α4β7-Integrin therapy on T cells and clinical parameters in Inflammatory Bowel Disease: Exploring potential therapy outcome predictors (Deutsche Übersetzung: Der Einfluss der anti-α4β7-Integrin Therapie auf T-Zellen und klinische Werte in chronisch entzündlicher Darmerkrankung: Die Suche möglicher Therapieprädiktoren)“ selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren/innen beruhen, sind als solche in korrekter Zitierung kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) werden von mir verantwortet.

Ich versichere ferner, dass ich die in Zusammenarbeit mit anderen Personen generierten Daten, Datenauswertungen und Schlussfolgerungen korrekt gekennzeichnet und meinen eigenen Beitrag sowie die Beiträge anderer Personen korrekt kenntlich gemacht habe (siehe Anteilserklärung). Texte oder Textteile, die gemeinsam mit anderen erstellt oder verwendet wurden, habe ich korrekt kenntlich gemacht.

Meine Anteile an etwaigen Publikationen zu dieser Dissertation entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem/der Erstbetreuer/in, angegeben sind. Für sämtliche im Rahmen der Dissertation entstandenen Publikationen wurden die Richtlinien des ICMJE (International Committee of Medical Journal Editors; www.icmje.og) zur Autorenschaft eingehalten. Ich erkläre ferner, dass ich mich zur Einhaltung der Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis verpflichte.

Weiterhin versichere ich, dass ich diese Dissertation weder in gleicher noch in ähnlicher Form bereits an einer anderen Fakultät eingereicht habe.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer

unwahren eidesstattlichen Versicherung (§§156, 161 des Strafgesetzbuches) sind mir bekannt und bewusst.“

02.11.2023

Datum Unterschrift

7.2 Anteilserklärung an etwaigen erfolgten Publikationen

Konstantin Fritz hatte folgenden Anteil an den folgenden Publikationen:

Publikation 1: Horn V*, Cancino C*, Steinheuer L*, Obermayer B*, **Fritz K**, Nguyen A, Bösel B, Burns M, Schulz A, Mantzivi E, Lissner D, Conrad T, Mashreghi M, Sonnenberg E, Beule D, Flatz L, TRR241 IBDome Consortium, Weidinger S, Mei H, Siegmund B, Thurley K, Hegazy: A Multiparametric profiling and machine learning identify proliferating circulating effector CD4 T cells as predictors of vedolizumab failure in inflammatory bowel disease, [status 25 October 2023: submitted; can be found under https://biorxiv.org/cgi/content/short/2023.10.01.560386v1]; the first four authors marked with a star shared the first authorship.

Beitrag im Einzelnen:

- Rekrutierung der Patient*innen und Dokumentation sowie Analyse ihrer klinischen Parameter
- Isolation von PBMCs und Serum aus Patientenblut
- Analyse der durchflusszytometrischen Daten
- Graphiken, zu denen Konstantin Fritz beigetragen hat:
    - Figure 1b, f, i
    - Extended Figure 1
    - Extended Figure 8 a and b
    - Technical Supplementary Figure 1
    - Technical Supplementary Figure 8

[redacted]

Unterschrift, Datum und Stempel des/der erstbetreuenden Hochschullehrers/in

[redacted]

Unterschrift des Doktoranden/der Doktorandin

7.3 Curriculum vitae – Konstantin Fritz

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

7.4 List of publications

Horn V*, Cancino C*, Steinheuer L*, Obermayer B*, **Fritz K**, Nguyen A, Bösel B, Burns M, Schulz A, Mantzivi E, Lissner D, Conrad T, Mashreghi M, Sonnenberg E, Beule D, Flatz L, TRR241 IBDome Consortium, Weidinger C, Mei H, Siegmund B, Thurley K, Hegazy: A Multiparametric profiling and machine learning identify proliferating circulating effector CD4 T cells as predictors of vedolizumab failure in inflammatory bowel disease, [status 25 October 2023: submitted; can be found under https://biorxiv.org/cgi/content/short/2023.10.01.560386v1]

The first four authors marked with a star shared the first authorship.

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7.6 Bescheinigung des akkreditierten Statistikers

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Bescheinigung

Hiermit bescheinige ich, dass *Konstantin Fritz* innerhalb der Service Unit Biometrie des Instituts für Biometrie und klinische Epidemiologie (iBikE) bei mir eine statistische Beratung zu einem Promotionsvorhaben in Anspruch genommen hat. Folgende Beratungstermine wurden wahrgenommen:

- 02.09.2021
- 24.03.2022

Folgende wesentliche Ratschläge hinsichtlich einer sinnvollen Auswertung und Interpretation der Daten wurden während der Beratung erteilt:

- Gruppenvergleich mittels nicht-parametrischer Tests
- Problematik von Overfitting erläutert
- Allgemeine Hinweise zur Form und Darstellung der Ergebnisse

Diese Bescheinigung garantiert nicht die richtige Umsetzung der in der Beratung gemachten Vorschläge, die korrekte Durchführung der empfohlenen statistischen Verfahren und die richtige Darstellung und Interpretation der Ergebnisse. Die Verantwortung hierfür obliegt allein dem Promovierenden. Das iBikE übernimmt hierfür keine Haftung.

Datum: 19. September 2023

[redacted]