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Department of General Surgery
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Characterisation of the antibody response to commensal bacteria in Crohn´s disease

submitted for the fulfilment of the requirements for the degree of

Bachelor of Science (BSc.)

submitted by
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Table of Contents

1. Introduction

1.1. Inflammatory Bowel Diseases

Inflammatory bowel diseases (IBD) are defined as chronic, recurrent inflammatory conditions that mainly affect the gastrointestinal tract, but can also affect extraintestinal sites. Ulcerative colitis (UC) and Crohn´s disease (CD) are the two main clinical and pathological subtypes of IBD (Kaser et al. 2010). UC primarily affects the colonic mucosa without transmural inflammation and causes a continuous ascending inflammation extending from the rectum (Ord s et al. 2012). CD can affect the entire gastrointestinal tract and results in transmural inflammation, defined as involvement of all layers of the intestinal wall. In contrast to the mainly ascending inflammation in UC, CD patients often show skip lesions with healthy parts of the intestine mixed in between inflamed areas (Xavier and Podolsky 2007).

Clinically, IBDs display a versatile symptomatic spectrum. Abdominal pain, fatigue, unintended weight loss, diarrhea and bloody stool represent common clinical symptoms (Feuerstein and Cheifetz 2017). In the past decade, the incidence and prevalence of IBDs has markedly increased (Kaplan and Ng 2017). While some factors contributing to this rising number include westernized-lifestyle associated environmental triggers (Carreras-Torres et al. 2020), the pathogenesis of the disease is incompletely understood and characterised by a complex interaction of various factors (Figure 1)(Sartor 2006).

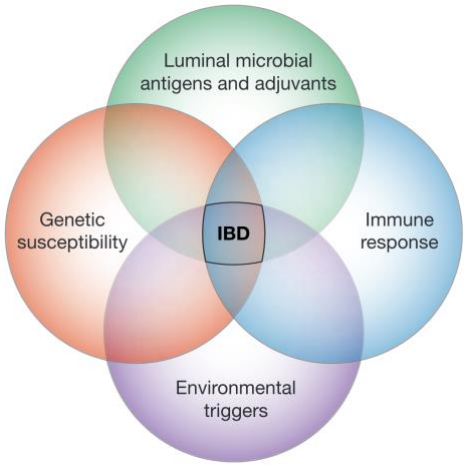

Figure 1. The interplay of different factors triggering the onset of IBD. The development of chronic inflammation in the intestine is characterized by the interaction of the genetic susceptibility to the disease, immune response of the innate and adaptive immune system to the gut microbiota and environmental triggers (Sartor 2006).

In the treatment goals of IBD has been a radical change. Where the main goal in the past was remission and control of symptoms, the focus has shifted to induce mucosal healing with a decrease in intestinal inflammation and the restoration of a healthy gut mucosa, to significantly improve the quality of life of IBD patients (Cai et al. 2021). However, complete mucosal healing, the ideal treatment response, cannot always be achieved and the lifetime risk for patients with IBD, and especially patients with CD, to require surgery is high (Tsai et al. 2021). To date, immunosuppressive therapies aim to (unspecifically) dampen inflammation, control symptoms, induce and, ultimately, sustain remission (Torres et al. 2020). In recent years, the armamentarium of drugs has expanded to aminosalicylates, steroids, immunomodulators and biologics (Torres et al. 2020, Raine et al. 2022). Thus, as a significant fraction of patients does not respond to the accessible treatment options or suffers from a loss of response to established treatments (Kennedy et al. 2019), the necessity to identify new therapeutic strategies arises. Furthermore, to enable a personalised approach with tailored treatment to the individual patient further identification of biomarkers for the disease outcome and treatment response as well as even more precise tests are necessary (Noor et al. 2020).

1.2. The role of B cells and Immunoglobulins (Igs) in inflammation

As fundamental components of humoral adaptive immunity, B cells can mature and ultimately secrete antibodies which are able to protect against a huge variety of pathogens. The protection can be both, short as well as long lived, and is achieved by their capability of recognizing an almost infinite number of unknown antigens. The origin of naïve B cells is found in the bone marrow, where they develop from haematopoietic progenitor cells under the influence of various stimuli and factors (Pieper et al. 2013). To initiate the production of antibodies, naïve B cells are activated to differentiate and maturate by the encounter of a foreign antigen, specific to their B cell receptor (BCR), which is located on the surface of naïve B cells and represents a membrane-bound form of immunoglobulin. After the following maturation and differentiation processes in secondary lymphoid organs (explained in section 1.3.), each terminally differentiated B cell produces immunoglobulins of a single specificity. This creation of antibodies, which bind to antigens with high specificity, represents the central role of B cells in the setting of adaptive immunity (Treanor 2012, Murphy and Weaver 2016).

Depending on the localization of B cells, their response can vary and different immunoglobulin isotypes can be produced, with distinct effects on the immune response. Specifically, five different isotypes of human Ig classes, namely IgG, IgM, IgD, IgE and IgA, exist. All classes have similarities in their basic structure, but also distinct differences in the function as well as structure. In detail, the basis for the distinction are structural differences in the Fc fragments of the different classes (Mix et al. 2006).

Most important for mucosal immunity is IgA, which is predominantly secreted into the intestinal lumen and is therefore present on mucosal barriers. Thus, its most important function is to opsonize and neutralize pathogens, ultimately limiting the invasion of bacteria (MacPherson et al. 2008, Salim and Söderholm 2011). IgG, which is the most abundant isotype in extracellular fluid and blood, is mainly opsonizing antigens to mediate phagocytosis by neutrophils and macrophages, and is capable of activating the complement system via the classical pathway (Dunkelberger and Song 2010, Abbas et al. 2021).

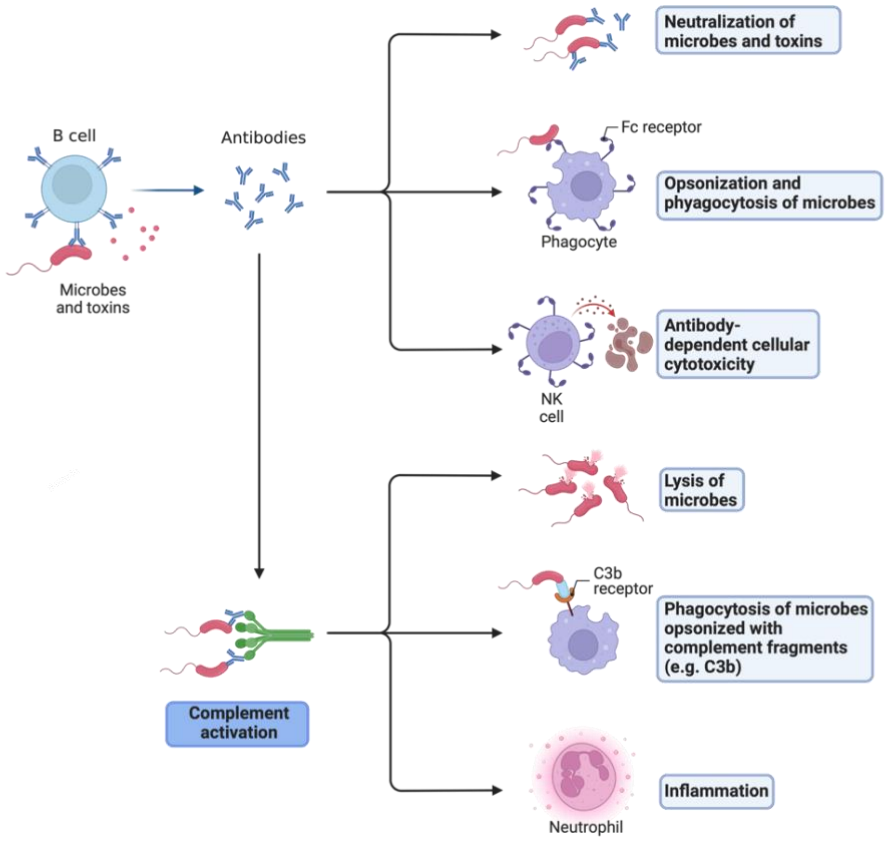

Figure 2 Effector functions of antibodies (Figure created using BioRender.com BioRender.com).

1.3. Germinal centers (GCs)

Germinal centers are transient, microanatomical structures formed by proliferating B cells in the follicles of secondary lymphatic tissues, being responsible for the affinity maturation and development of naïve B cells (Eisen 2014). In the initiating process of the formation of GCs, naïve B cells are activated by the encounter with an antigen. Microanatomically, GCs consist of a dark zone and a light zone. The dark zone represents the location of proliferation and somatic hypermutation (SHM) of naïve B cells (Victora and Nussenzweig 2022). SHM causes diversification of the immunoglobulin variable region through introducing point mutations in the heavy or light chain variable region gene segments, which results in the expression of closely related antibodies that differ in their ability to bind the immunizing antigen. Hence, antibody mutants with lower affinity to the antigen or those with autoreactive specificities are displaced by competitors with higher affinity and undergo apoptosis (Mayer et al. 2017). Notably, B cells with high binding affinity migrate into the light zone, where they may undergo immunoglobulin class-switch recombination (CSR) and further differentiation. CSR enables B cells to modify their immunoglobulin isotype, to produce antibodies with altered effector functions (Klein and Dalla-Favera 2008). In addition, there is also the possibility of light zone-dark zone recirculation, leading to further improved affinity to the antigen (de Silva and Klein 2015). Eventually, a smaller fraction of B cells differentiates into memory B cells, or antibody-secreting plasma cells, both expressing a highly selected antibody repertoire (Klein und Dalla-Favera 2008, Victora and Nussenzweig 2022). Upon antigen re-encounter, memory B cells can very quickly differentiate into short-lived plasma cells, thus directly leading to the production of antibodies with high antigen affinity (McHeyzer-Williams and McHeyzer-Williams 2005).

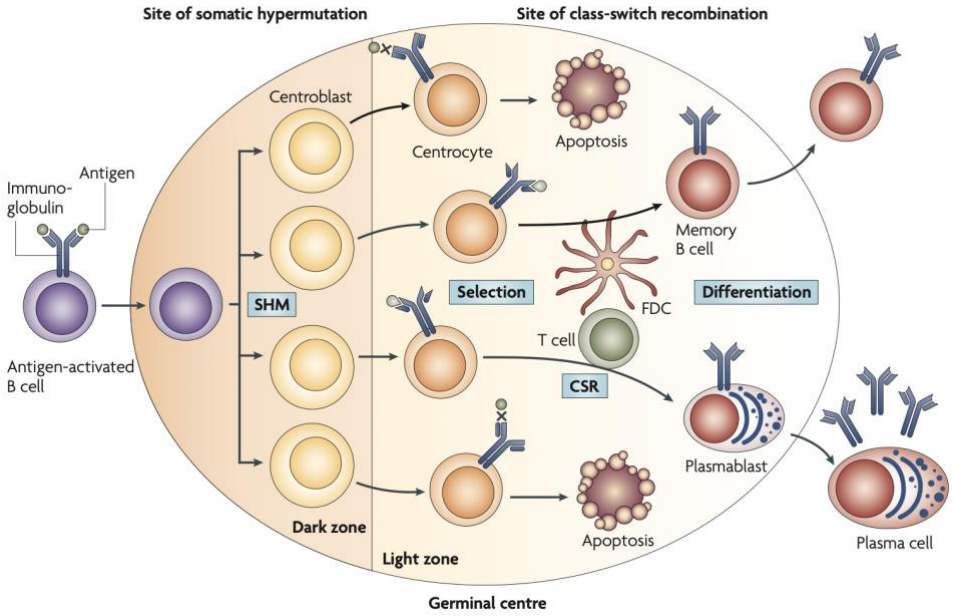

Figure 3 The microenvironment of Germinal centers (Klein und Dalla-Favera 2008).

1.4. Fc-gamma receptors (FcγRs)

While the variable region of Igs recognizes a specific antigen, their constant regions, which dependent on their respective isotype, are recognized by receptors which are expressed by many immune cells (Daëron 1997). One class of these receptors are Fc γ receptors (FcγRs), which recognize IgG via their Fc portion. To date, four classes of the human FcγRs have been identified. FcγRI, FcγRIII and FcγIV act as activating receptors on the expressing immune cell, while FcγRIIB, results in inhibiting signals (Nimmerjahn and Ravetch 2006). The ratio between activating and inhibitory FcγRs (A:I ratio) has shown to be modulating the immune response. This modulation can either lead to activation or inhibition of the immune cells. Predominantly activating signals can lead to additional pro-inflammatory mechanisms and cell activation, whereas receiving predominantly inhibitory signals can inhibit further cell activation and pro-inflammatory responses (Castro-Dopico and Clatworthy 2019). Other functions of FcγRs include antigen presentation, dendritic cell maturation mediated by immune responses, regulation of B-cell activation, and plasma cell survival (Nimmerjahn and Ravetch 2006). These findings show, why FcγRs and the mechanisms which they are responsible for, could be relevant targets for novel therapeutics (Nimmerjahn and Ravetch 2008).

1.5. Association of anti-commensal IgG and intestinal Inflammation in IBD

Intestinal Inflammation, caused by CD and UC, leads to increased bacterial translocation that results in commensal gut bacteria, and thus antigens, passing through the intestinal barrier and stimulating an immune response. These responses can either be physiological, in case of a short-term translocation, or pathological, in case of continuous inflammation and persistent severe inflammation (Neurath 2014). In homeostatic conditions, there is a balance between the intake of essential nutrients and the exposure of the innate and adaptive immune system to a wide variety of antigens. Hence, if too many commensals pass through this damaged mucosal barrier, as in IBD, a cascade of immune responses with resulting proinflammatory mechanisms are triggered and sustained (Salim and Söderholm 2011).

While several different mechanisms of pro-inflammatory responses have been described in IBD, the role of B cells, and thus humoral immunity, remains controversial. Early work recognized that patients with IBD develop antibody against commensals, and that IgG is found in the stool in patients with IBD (Macpherson et al. 1996). While these findings have been published a while ago, more recent research aims on defining the pathophysiological role.

Alexander et al. (2021) investigated, whether there is an increase in IgG antibodies against microbiota flagellins in the serum of patients with CD and UC compared to healthy controls and indeed, the study found a higher amount of IgG directed against flagellins in sera from CD patients.

In another recently published study, the cellular landscape of inflamed ileum lesions in CD was mapped using single cell sequencing, and unique molecular signature was found in inflamed intestinal sections. Besides cellular subsets that have previously been thoroughly investigated, the study found a high presence of B cells, IgG^+ plasma cells (Martin et al. 2019).

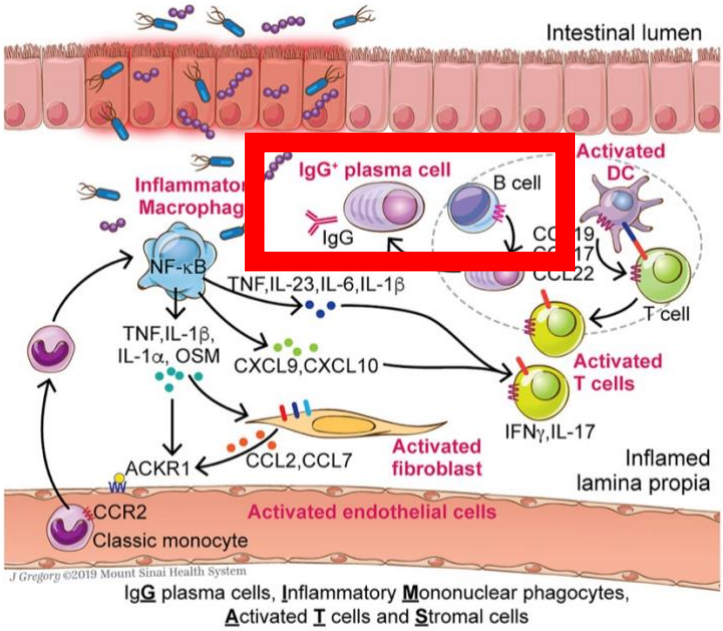

Figure 4 Cellular landscape of inflamed lesions in CD (modified) (Martin et al. 2019)

The findings of an activated B cell response, and isotype switch to IgG, are in line with results obtained in patients and animal models for UC. In a study by Castro-Dopico et al. (2019), the authors investigated B cell responses in UC, as mutations in FcγRs are known to be protective against UC (Jostins et al. 2012). In their study, stool samples of patients with UC and healthy household controls (HHC) were analysed to determine the amount of IgG- and IgA1/2-bound luminal commensals. As expected, a significantly higher number of IgG-bound commensals in stool sample of patients with UC, compared to HHC, were found recognized. In contrast to the findings in IgG-bound commensals, the number of IgA1/2-bound commensals in samples of UC patients was not significantly higher compared to HHC, as this immunoglobulin isotype is predominantly present in mucosal areas. Subsequently, also a correlation with the clinical activity index, a disease severity score, was performed and patients with the highest severity score showed higher levels of IgG-bound commensals in their stool sample. To further focus on the cellular consequences of the higher proportion of IgG-bound commensals, investigation on the FcγR receptor were conducted and additional proinflammatory mechanisms were found at a higher FcγR activating to inhibiting (A:I) ratio, suggesting that the ratio influences the magnitude of intestinal inflammation. Hence, in UC the activation of FcγR through IgG seems to result in the aggravation of the disease (Castro-Dopico et al. 2019), while the role of anti-commensal IgG antibodies in sustaining CD is less clear.

2. Aim of the thesis

As B cells are the precursors to plasma cells, and receptor rearrangement in germinal centers results in antigen-specific immunoglobulins, further characterization of this population can provide valuable information in the initiation of the pathological antibody response in Crohn´s disease. Within this thesis, the aim was to characterise potential changes in size and quantity of germinal centers in mesenteric lymph nodes draining diseased intestinal segments of patients with Crohn´s disease and compare them to germinal centers from lymph nodes draining unaffected intestinal segments by immunohistochemistry.

Furthermore, while in previous studies IgG antibodies directed against commensals have been detected in serum samples of patients with Crohn´s disease, it is less clear whether these antibodies can always be found in patients undergoing surgery. Hence, in order to identify a possible difference in the relative frequency of bound IgG and IgA on commensal bacteria between stool samples from Crohn´s disease patients and healthy controls, microbial flow cytometry has been established.

3. Material & Methods

3.1. Buffers and solutions

Citrat Buffer	
Trisodium Citrat (Sigma-Aldrich, Germany)	10 mM
Aqua dest.	500 mL
Tween 20 (Sigma-Aldrich, Germany)	0.25 mL
Adjust to pH 6.0 with 2 N HCl	
Phosphate Buffered saline + Tween 20 (PBS-T)	
1x PBS (Thermo Fisher Scientific, USA)	
Tween 20 (Sigma-Aldrich, Germany)	0.1% (v/v)
Fluorescence-activated cell scanning (FACS) Buffer	
1x PBS	
BSA (Bovine Serum Albumin; Serva GmbH, Germany)	2%
FIX Buffer	
1x PBS	
BSA	2%
Formaldehyde	0.5%
Washing Buffer	
1x PBS	
Fetal Calf Serum (FCS; Linaris, Germany)	2%
EDTA (0.5 M, pH 8.0; Invitrogen, USA)	1 mM
Crystal violet solution	
Crystal violet	0.05%
Ethanol	2%

3.2. Sample collection

Blood and stool samples were collected from patients with CD following informed consent and previous approval by the Medical University of Vienna's ethics committee (EK# 1915/2021) at the time of surgery due to CD complications. Additionally, tissue samples from inflamed and uninflamed mesenteric lymph nodes of CD patients were collected. As control, serum from unrelated healthy volunteers was used.

3.3. Immunohistochemistry (IHC)

Inflamed and uninflamed mesenteric lymph nodes from Crohn´s disease patients had been previously obtained and were formalin-fixed and paraffin-embedded. For sectioning, specimen were cut into 4-µm-thick histological sections, using a sliding microtome (SM2000R, Leica, Germany). Before IHC, suitable slides were preselected. For slide preparation, selected slides were put into the oven at 60°C overnight or at least for an hour. Afterwards, slides were kept at room temperature for 10 minutes. Deparaffinization and rehydration with a graded alcohol series starting from Xylene (Sigma-Aldrich, Germany) to 100%, 80%, 70%, 50% ethanol (Ethanol absolute, VWR Chemicals, USA; diluted with distilled water) followed. Rehydration was finished with two changes of distilled water. To block endogenous peroxidase, slides were incubated for 10 minutes in the dark at room temperature with 0.3% hydrogen peroxide (H_2O_2). Therefore, hydrogen peroxide 30% (Merck, Germany) was diluted 1:10 with distilled water. After washing with 1x PBS at room temperature for 3 minutes twice, slides were put into 10 mM Tri-sodium citrate buffer (pH 6.0) and cooked in a tabletop steam autoclave (CertoClav, Germany) to a temperature of 119°C and subsequently cooled back down to 70°C, for heat induced antigen retrieval. Once tissue on the slide was outlined with a liquid blocking pen and washed with 1 x PBS containing 0.1% Tween 20 (PBS-T) for 3 minutes, blocking of non-specific binding was ensured by incubation with normal goat serum 2.5% (Vector Laboratories, USA) for 20 minutes at room temperature. To identify an optimal dilution of the primary antibody, purified anti-human CD19 (BD Pharmingen, USA), different dilutions were tested. From the dilutions of 1:50, 1:100 and 1:150, a 1:100 dilution of purified anti-human CD19 in PBS-T, containing 1% Goat serum (Dako, USA), proved to be most suitable. The negative control was carried out without primary antibody. Incubation of the primary antibody was performed overnight at 4°C in the humidity chamber. Prior to the incubation with the secondary antibody, sections were washed twice in PBS-T at room temperature for 3 minutes. The

secondary antibody, goat-anti-mouse IgG (Vector Laboratories, USA), was incubated for 1 hour in the humidity chamber at room temperature. Afterwards, sections were again washed twice in PBS-T at room temperature for 3 minutes. To visualize the antigen-antibody interaction, 3,3´-diaminobenzidine (Liquid DAB+ Substrate Chromogen System, Dako, USA) was applied. Once brown colouring was sufficient, sections were placed in a cuvette with distilled water, to stop the reaction. For nuclear counterstaining, slides were stained with Gill´s hematoxylin (Merck, Germany) for 2 minutes.

After brief rinsing with distilled water, slides were collected in Scott´s bluing solution (Morphisto, Germany). Dehydration was performed with a graded alcohol series starting from 70% to 80%, 96% and 100% ethanol. Subsequently, sections were collected in n-butyl acetate (Sigma-Aldrich, Germany) and mounted with Entellan™ (Sigma-Aldrich, Germany).

Of each slide, microscopic pictures were taken with 10x magnification using the AxioScope A1 microscope (Zeiss, Germany). The number of pictures taken per slide depended on the size of the mesenteric lymph node. The quantification of the germinal centers was performed per High Power Field (HPF). The diameter of GCs was counted using the ZEN Blue Software. With the line tool, the diameter of each GC was determined. Afterwards, data was entered into the Prism 8 (GraphPad) software for statistical analysis and visualization. Following evaluations were performed using Zeiss ZEN (blue edition) and for statistics GraphPad Prism 8.

3.4. Microbial flow cytometry

3.4.1. Serum preparation

After blood sample collection in serum tubes with separation gel (Greiner Bio-One, Germany), samples were stored at room temperature for 30 minutes to enable clotting. For clot removal, centrifugation at 1,000 rpm for 10 minutes at 4°C (ROTANTA 460 Robotic Centrifuge, Hettich, Germany) was necessary. Afterwards, serum was handled on ice. Serum was apportioned into 500 µL aliquots and stored at 4°C for immediate usage or stored at -80°C if it was not used immediately.

3.4.2. Stool sample preparation

Before processing, stool samples were stored at 4°C. To start the washing steps, 1x PBS was added to the stool sample. The exact amount of added sterile 1x PBS depended on the weight of the processed stool sample. Through shaking for 1-2 minutes, faecal contents were

homogenised. After transfer of the homogenised stool sample into a 15 mL Falcon tube (Corning, USA), centrifugation at 1,000 rpm for 5 minutes at 4°C followed, to remove large aggregates. The supernatant was transferred into another 15 mL Falcon tube and again briefly centrifuged at 1,000 rpm for 5 minutes at 4°C. The resulting supernatant was transferred into 2 mL Eppendorf tubes and centrifuged for 5 minutes at 10,000 g at 4°C (Microfuge 22R Centrifuge, Beckman Coulter, USA), to receive a bacterial pellet. After the supernatant was discarded, the pellet was resuspended in 1 mL 1x PBS.

3.4.3. Crystal violet staining

To validate the presence of bacteria in the bacterial pellet, the bacterial pellet was stained with crystal violet solution (0.05% Crystal violet, 2% ethanol). Before staining, a small amount of bacterial pellet was air dried on a slide. After fixation of the sample through briefly passing it over the Bunsen burner for three times, 60 seconds of incubation with some droplets of Crystal violet solution followed. Subsequently, the slide was washed with distilled water, air dried and assessed under the microscope without cover glass.

3.4.4. Checking rate of detected events per second in the flow cytometer

For identification of the optimal dilution of bacterial solution, different dilutions of the solution were tested. 1:10, 1:50 and 1:100 dilutions with 1x PBS were prepared. To receive a pellet, centrifugation for 8 minutes at 10,000 g at 4°C in a table-top centrifuge followed. Supernatant was removed, bacteria were fixed in 250 µL fix buffer (2% BSA, 0.5% formaldehyde) and subsequently transferred into specific flow cytometry tubes. These additional quality control steps were performed as ideally, not be more than 2800 events per second should be recorded to allow distinction between events and avoid doublets/bacterial conglomerates due to an increased density of bacteria in the solution.

3.4.5. FACS staining with SYBR Green

After determining an adequate dilution of bacterial solution, 1.5 mL Eppendorf tubes containing 100 µL aliquots were prepared. The number of Eppendorf tubes was according to the required number of tests for the measurement with the flow cytometer. Subsequently, 10 µL of 1:100 pre-diluted SYBR Green (Thermo Fisher Scientific) were added into each Eppendorf tube, to

receive a 1:1000 dilution of SYBR Green. After vortexing, samples were incubated with SYBR green in the dark at 4°C for 15 minutes. Finally, samples were centrifuged for 8 minutes at 10,000 g at 4°C in a table-top centrifuge to receive a pellet.

3.4.6. Serum incubation

To enable binding of IgA and IgG antibodies directed against commensal bacteria, supernatant was removed and the bacterial pellet was resuspended in 49 µL 1x PBS. Then, 1 µL patient serum was added to receive a 1:50 dilution. After incubation for 20 minutes in the dark at 4°C, specimen were diluted up to 1 mL with FACS Buffer for washing and centrifuged at 8,000 g for 5 minutes at 4°C. The washing step was performed twice to ensure adequate removal of excess serum sample and subsequent unspecific binding.

3.4.7. Antibody staining

After serum incubation, bacterial pellets were resuspended in 250 µL FACS buffer and incubated with 20 µL anti-human IgG-APC (BD Pharmigen, USA) and 1 µL anti-human IgA-VioBlue (Milentyi Biotec, Germany). After incubation for 30 minutes in the dark at 4 °C, samples were centrifuged at 8,000 g for 5 minutes at 4°C. Subsequently, specimen was filled up to a volume of 1 mL with FACS Buffer and centrifuged at 8,000 g for 5 minutes at 4°C. The supernatant was removed, the sample was again filled up to 1 mL with FACS Buffer and centrifuged at 8,000 g for 5 minutes at 4°C. Once the supernatant was removed, stained bacteria were fixed in Formaldehyde before FACS analysis by adding 250 µL Fix Buffer, and transferred into FACS tubes for subsequent analysis. Acquisition was performed on a flow cytometer (DxFLEX, Beckman Coulter, USA) using the CytExpert software (Beckman Coulter, USA).

3.5. Flow cytometry of peripheral blood mononuclear cells (PBMCs)

3.5.1. Isolation of PBMCs

As quality control of the used IgG antibodies and to verify their specificity, PBMCs were used to measure IgG^+ B cells. For the isolation of peripheral blood mononuclear cells (PBMCs) from EDTA blood, whole blood was distributed into 50 mL Falcons (Corning, USA) and subsequently 15 mL of washing buffer (1x PBS, 2% FCS, 1 mM EDTA) was added. Next,

whole blood was mixed with 13 mL of Ficoll-Paque Plus (GE Health Bioscience AB, Germany), and samples were centrifuged at 1,000 g for 10 minutes at room temperature (Allegra® X-12R Centrifuge, Beckman Coulter, USA). Samples were carefully taken from the centrifuge and as much of plasma as possible was removed. The interphase containing PBMCs was taken off with a Pasteur pipette and transferred into a fresh Falcon tube, and 50 mL of washing buffer was added. After another centrifugation step for 10 minutes at 300 g, supernatant was taken off and another 50 mL of washing buffer was added, followed by another centrifugation step at 300 g for 5 minutes. Once the supernatant was taken off, erythrocyte lysis was performed using 2 mL of VersaLyse Lysing Solution (Beckman Coulter, USA) and incubation for 5 minutes at room temperature. Lysis was stopped by adding up to 50 mL 1x PBS containing 2% fetal calf serum (FCS). After centrifugation at 300 g for 5 minutes, the resulting supernatant was taken off and PBMCs were washed another time with 1x PBS containing 2% FCS. Subsequently, the supernatant was discarded and the pellet resuspended in approximately 1 mL FACS Buffer (1x PBS/2% BSA). For cell counting, PBMCs were diluted 1:10 with 1x PBS in a 1.5 mL Eppendorf tube. Cell counting was performed using the Sysmex (XN-350, Sysmex, Japan).

3.5.2. FACS Staining of PBMCs

A total of 1 x 10^6 cells/tube was transferred into small FACS tubes and filled up to 1 mL with FACS Buffer. Centrifugation at 300 g for 5 minutes at 4°C followed as additional washing step. After the supernatant was aspired and discarded, PBMCs were resuspended in 50 µL FACS Buffer. Subsequently, 25 µL of antibody mix (Table 1) was added. Isotype control to the used antibodies was also implemented. No antibody was added to the unstained sample, instead 25 µL of FACS Buffer was added. Incubation for 30 minutes in the dark at 4°C followed. Having samples washed once with 1 mL FACS Buffer and centrifuged at 300 g for 5 minutes at 4°C, 200 µL of FACS Buffer were added and samples were immediately acquired at a DxFLEX flow cytometer. Subsequent analysis was performed using Kaluza Analysis Flow Cytometry Software.

Antibody (conjugate)
CD45 (eF450)
CD19 (FITC)
IgG (APC)

Table 1 Antibody mix for the FACS staining of PBMCs

3.6. Statistical analysis

Statistical analyses were conducted using GraphPad Prism 8 Software. For all statistical tests, an unpaired student´s t-test was performed.

4. Results

4.1. Quantification and measurement of germinal centers in mesenteric lymph nodes

A total of 10 slides of each, healthy and diseased paraffin-embedded mesenteric lymph nodes of patients with CD, were analysed to determine differences in size and number of germinal centers.

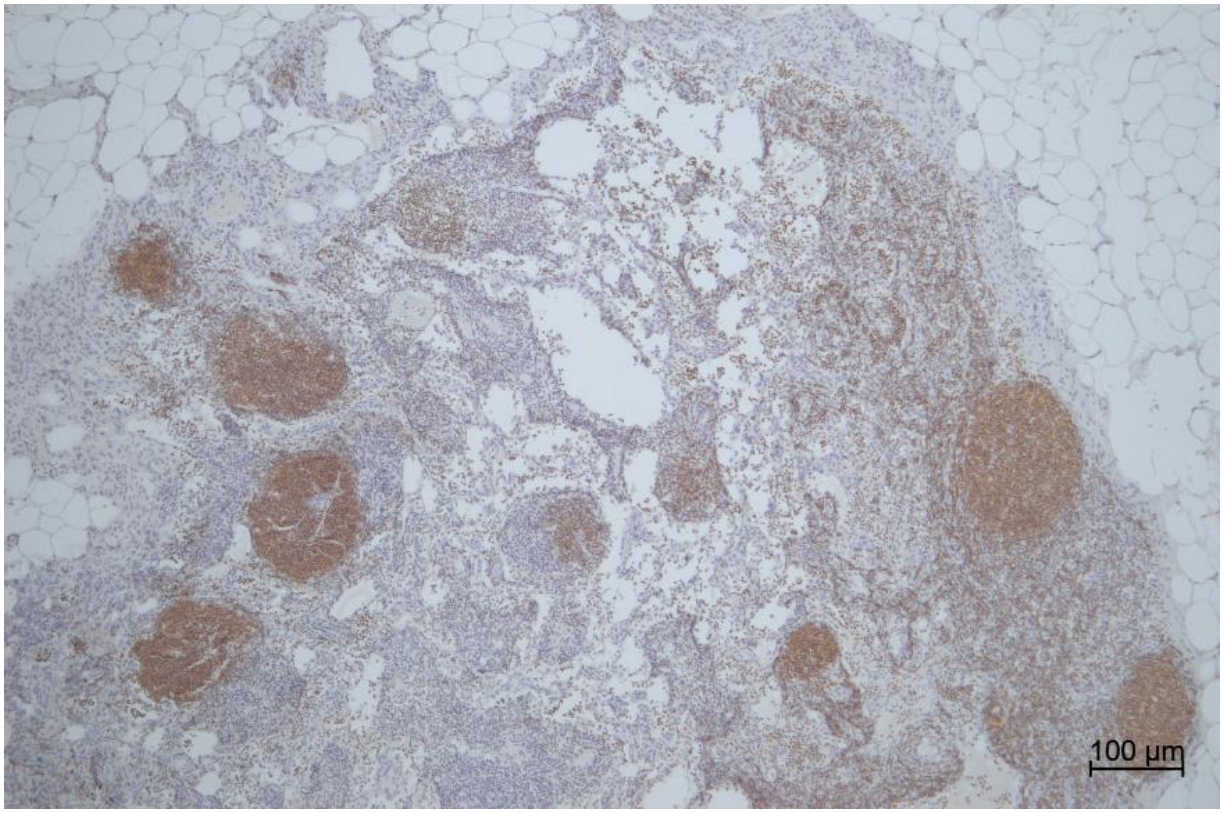

Figure 5 Representative image of a mesenteric lymph node draining a healthy intestinal segment. Immunohistochemical staining was performed for CD19, a B cell specific marker.

HPFs of mesenteric lymph nodes from uninflamed intestinal segments (n=13) and HPFs of mesenteric lymph nodes from inflamed intestinal segments (n=16) were counted from a total of n=10 patients each. Statistical analysis revealed no difference in the number of germinal centers between uninflamed and inflamed mesenteric lymph nodes per HPF (p=0.8516; Figure 6).

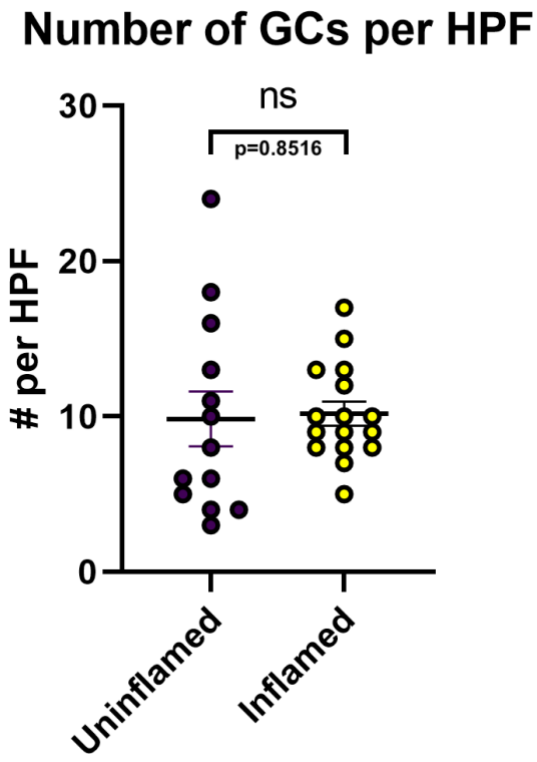

Figure 6 Number of GCs per HPF. A total of n=13 HPFs were analysed in mesenteric lymph nodes of intestinal sections without inflammation, while n=16 HPFs were analysed in mesenteric lymph nodes of intestinal sections with inflammation from a total of n=10 patients each. Unpaired student's t-test revealed no difference between groups (p=0.8516).

To assess whether the GC sizes differ, diameter of GCs were measured in the intestinal sections and a total of n=128 GCs were analysed in mesenteric lymph nodes from uninflamed intestinal sections and n=163 GCs in mesenteric lymph nodes from inflamed intestinal sections. When taken together, GCs of uninflamed mesenteric lymph nodes were on average smaller in size (113.06 µm ± 3.66 µm) than GCs from inflamed segments (149.33 µm ± 5.92 µm; $p<0.0001$). Data is shown in Figure 7.

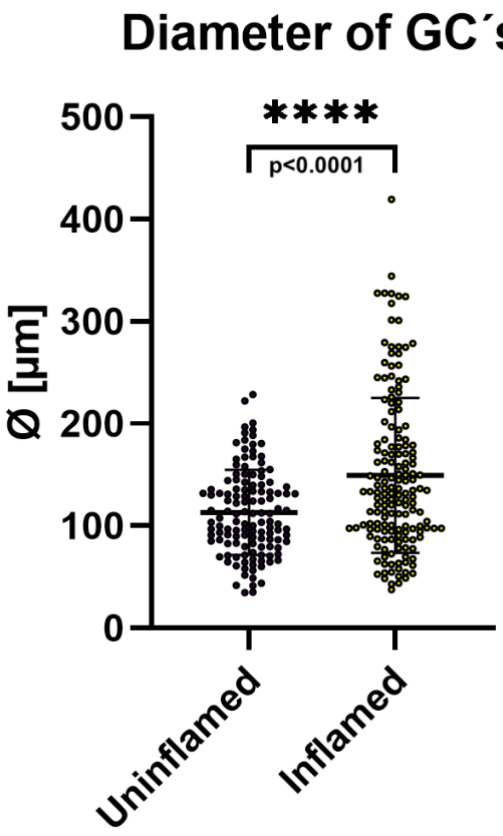

Figure 7 Diameter of GCs. (Uninflamed n=128; Inflamed n=163)

4.2. Establishment of microbial flow cytometry

4.2.1. Crystal violet staining of bacterial solution

While these results indicated that the local B cell immune response in inflamed intestinal sections could be directed towards commensal bacteria, it was next tested whether patients with CD develop antibodies towards commensals, as indicated in previously published studies. To first ensure the presence of bacteria in the bacterial pellet after washing, staining with crystal violet solution was performed. The staining with crystal violet served as a simplified Gram stain and stains all bacteria violet, as they are able to absorb the dye (Tripathi and Sapra 2022).

Microscopy without cover glass showed the presence of a large number of bacteria. Further identification of the exact bacterial species could not be conducted, but the observed shapes of bacteria indicated a mix of different strains. Besides bacteria, the bacterial pellet showed

the presence of very little residual fibres and other debris, and a high density of bacteria, warranting further dilution for the subsequent experiments.

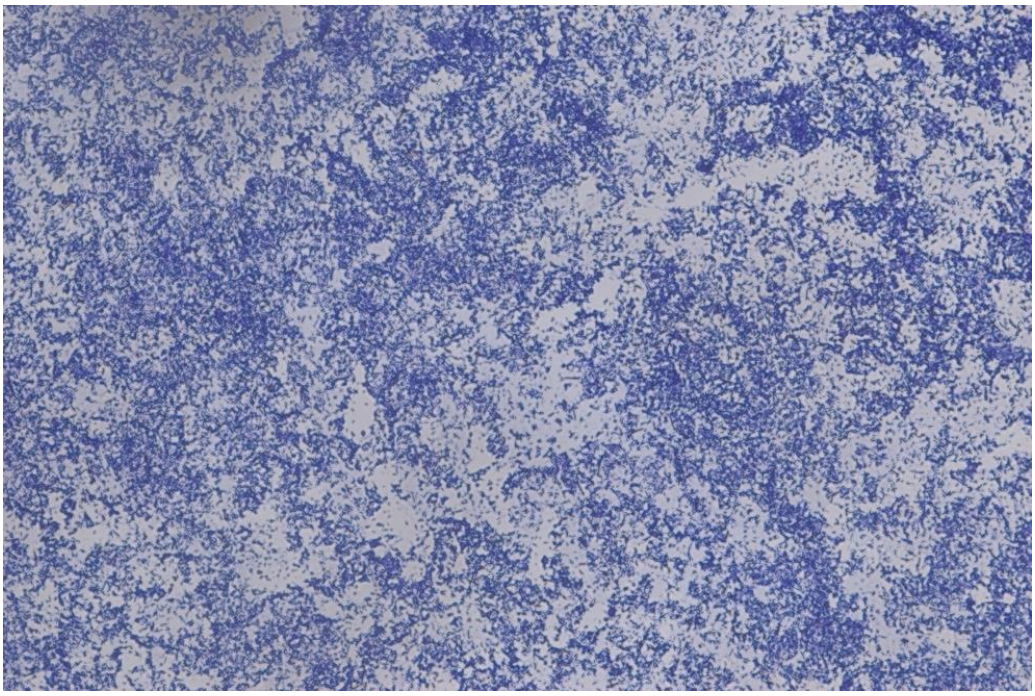

Figure 8. Representative microscopic image of a bacterial pellet (10x magnification).

4.2.2. Assessment of anti-commensal IgA and IgG antibodies via microbial flow cytometry

In the process of establishing the protocol for microbial flow cytometry, fresh stool samples from patients with IBD were used. Serum samples from patients with UC were used to establish the assay, as UC patients have reportedly the highest number of anti-commensal antibodies. To identify intact bacteria in FACS, the nucleic acid stain SYBR Green proved to be suitable for the identification of bacteria in the faeces. Prior to the antibody staining, an adequate SYBR Green dilution, which provided an appropriate signal in the flow cytometry measurement, was defined by testing different dilutions. Finally, a dilution of 1:1,000 delivered the best results, allowing reproducible measurements. Analysis by flow cytometry allowed to distinct $SYBR^+$ bacterial population from dead cells and debris, as shown in Figure 9b.

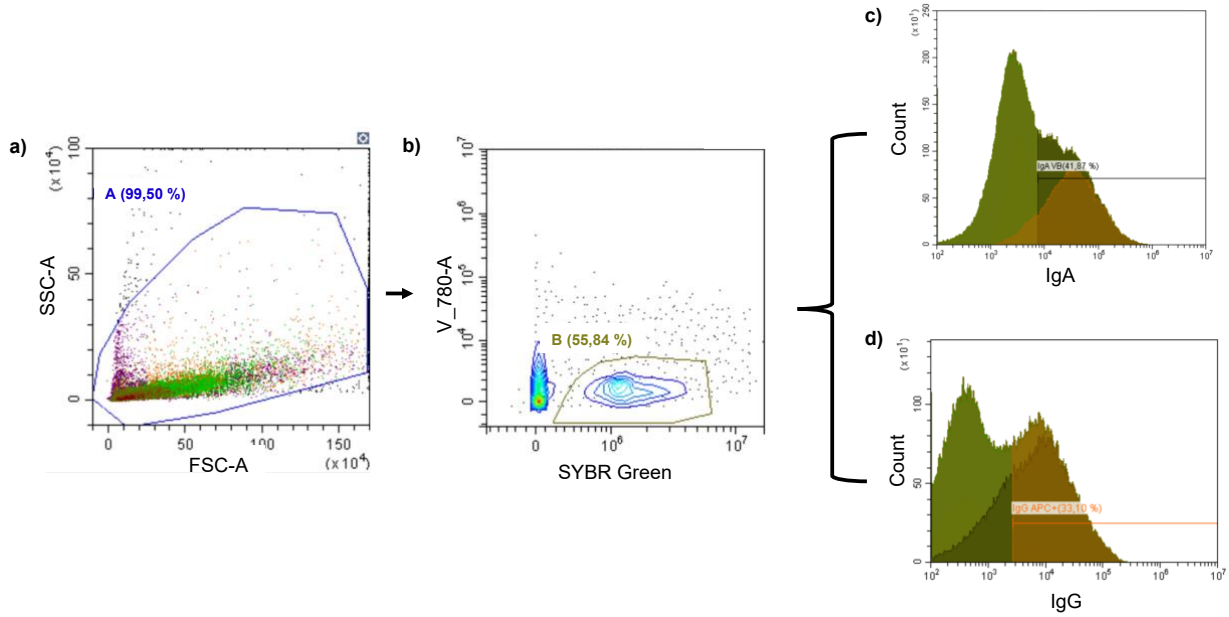

Figure 9 Gating strategy for microbial flow cytometry to identify IgG and IgA coated commensal bacteria. Having restricted the area based on forward scatter (FSC) and side scatter (SSC), SYBR$^+$ bacteria were used as target population (Gate B), as seen in Figure 9b. Subsequently, IgA$^+$ and IgG$^+$ bacteria were defined (Figure 9c and Figure 9d).

4.2.3. Flow cytometry of PBMCs

To verify specificity of the used IgG antibodies, PBMCs were used to measure IgG$^+$ B cells as quality control. For the staining, 1 x 10^6 cells per test were used, as recommended in the data sheet of the used IgG antibody. To be able to gate on the B cell fraction, CD45 (lymphocyte marker) and CD19 (B cell marker) were used. First, the gate was set on the CD45$^+$ lymphocytes, subsequently on the CD45$^+$CD19$^+$ B cells, in order to identify the CD45$^+$CD19$^+$IgG$^+$ B cell fraction. The used gating strategy can be seen in Figure 10. At least 500,000 cells were acquired per run. In Figure 10b, the IgG$^+$CD19$^+$ B cells are displayed in the upper right quadrant. As expected, a high fraction of B cells in peripheral blood expressed IgG, and specific binding of the antibody was thereby verified.

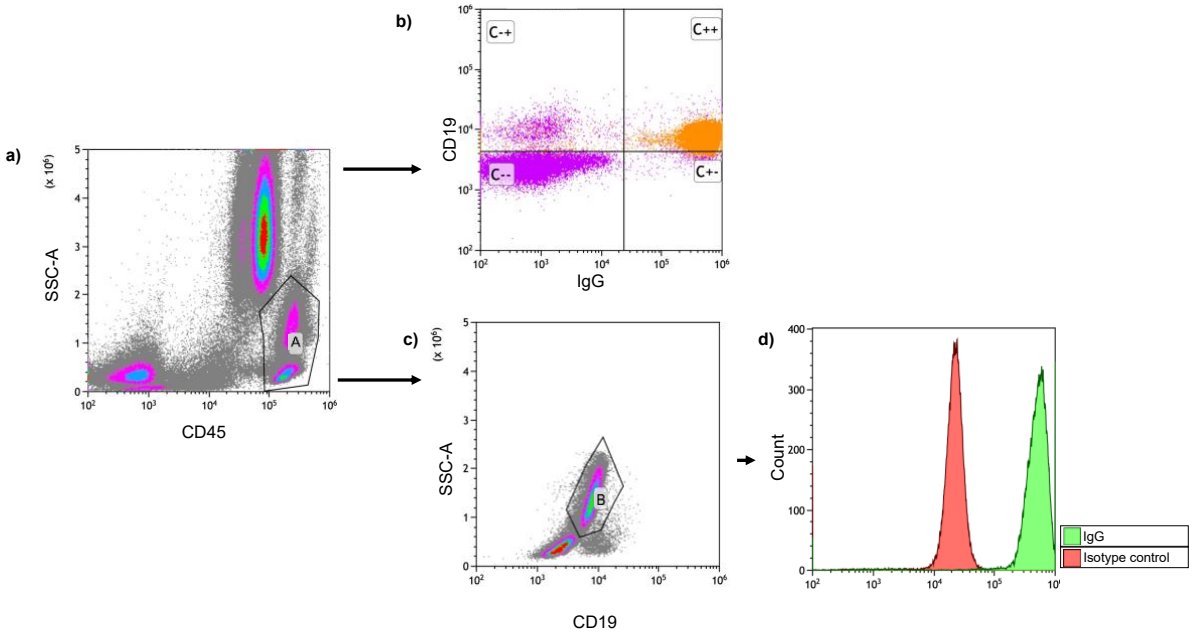

Figure 10 Gating strategy for identification of the PBMC population of interest.

Additionally performed isotype control ensured no unspecific binding of the antibody (Figure 10d). Comparison of the isotype control peak with the peak of the stained B cells revealed a clear shift of the peak.

4.3. Microbial flow cytometry

Once the protocol was established, serum samples of patients with IBD (n=15) and healthy controls (n=4) were incubated with stool samples of non-IBD patients to avoid background of secreted IgG. The IBD group consisted of one UC patient with acute inflammation, one patient diagnosed with a mixed type of UC, and 13 patients with CD.

Analysis of the collected flow cytometry data showed a higher fraction of IgG coated bacteria in IBD patients (HC: 6.23 ± 3.11 %; IBD: 15.07 ± 2.52 %), but differences did not reach statistical significance (p=0.1217, Figure 11a). Similarly, fractions of IgA coated bacteria did not show a difference between the groups (HC: 14.81 ± 4.85 %; IBD: 22.43 ± 4.93 %; p=0.2102, Figure 11b).

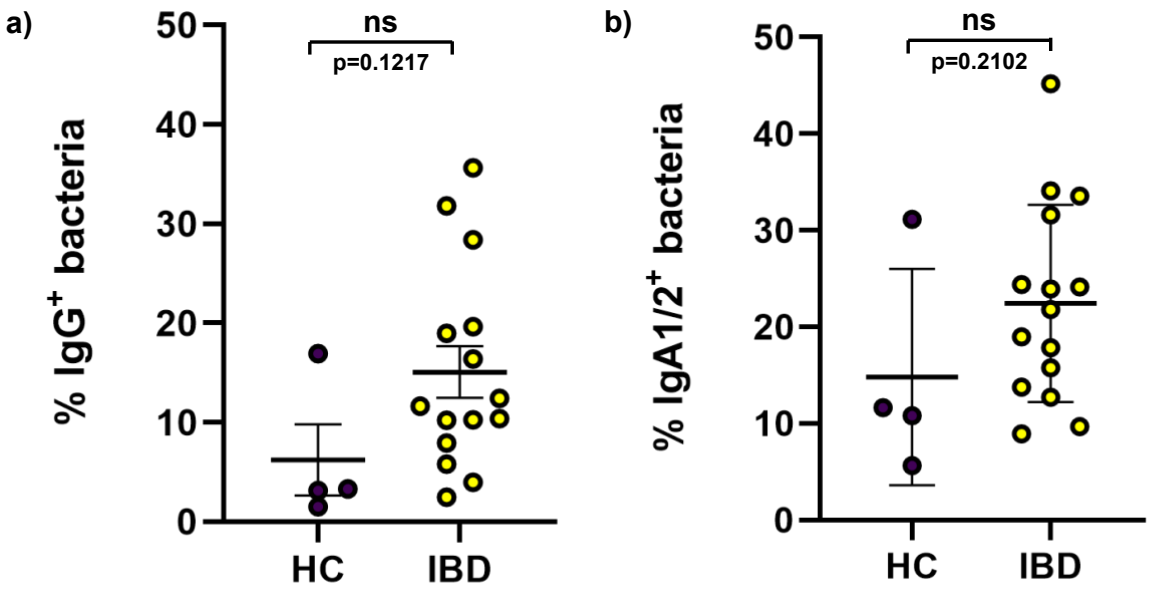

Figure 11 (a) IgG+ SYBR+ commensal bacteria and (b) IgA1/2+ SYBR+ commensal bacteria were analysed in healthy control group (n=4) and IBD patients (n=15) with an unpaired t-test.

In addition to the statistical evaluation of the IgG^+ and IgA^+ commensals, the number of double positive bacteria (recognized by IgA and IgG, respectively) was analysed to assess whether fractions of IgA and IgG bacteria differ in terms of recognized bacterial subsets. As shown in Figure 12, the unpaired t-test reported no significant difference between the IBD group and HC group (HC: 5.83 ± 2.85 %; IBD: 14.41 ± 2.38 %; p=0.1112).

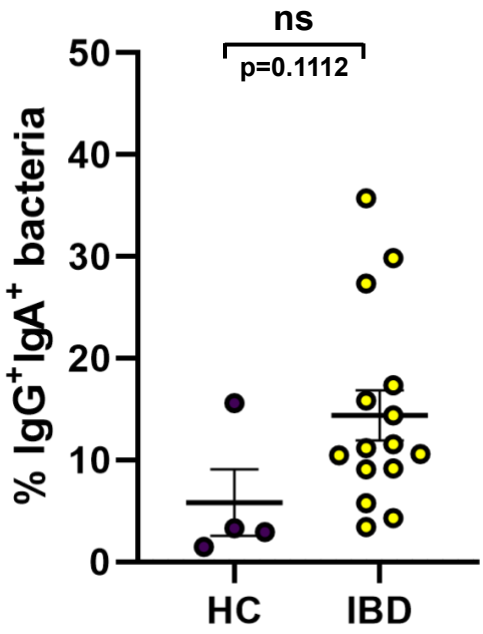

Figure 12 $IgG^{+}IgA^{+}$ commensal bacteria (HC n=4, IBD n=15).

5. Discussion

The study showed an aggravated B cell response in inflamed intestinal segments demonstrated by GC expansion, with an increased fraction of antibodies directed against commensals but no statistically significant difference. While IgG responses, that usually do not occur on mucosal surfaces, have been shown to be part of the inflammatory landscape within inflamed intestinal tissue of CD patients (Martin et al. 2019), its pathophysiological role has yet to be defined. In UC, past studies (Castro-Dopico et al. 2019) have already investigated whether there is a pathological antibody class switch from immunoglobulin class A, which is physiologically secreted on mucosal surfaces, to immunoglobulin class G, and they then further characterised the resulting downstream proinflammatory mechanisms mediated by this IgG production against commensal bacteria.

In this thesis, the main focus was to determine whether IgG antibodies against commensal bacteria are also produced in CD and if there is a difference in the relative frequency of IgA- and IgG-bound commensal bacteria. Since B cells develop in the GCs after activation through antigens and ultimately develop to antibody secreting plasma cells, it was investigated, whether changes in the microanatomical structure are detectable, as a result of the increased antigen stimulation. Indeed, the quantification and measurement of GCs revealed that GCs within inflamed mesenteric lymph nodes are not increased in number, but are significantly increased in size, compared to lymph nodes of uninflamed sites ($p<0.0001$). This could be a result of an increased B cell activation triggered by microbes breaking through the damaged intestinal barrier. Nevertheless, despite this promising result in the measurement of GCs, there are some limiting factors in the quantification of the GCs.

For this, light must be shed on the microanatomical localisation of GCs. Generally, they are microanatomically located in the marginal regions of lymph nodes. However, human (clinical) samples represent natural limitations, as they do not have such uniform model-like structure and each one differs from the other. Hence, to keep this limiting factor as small as possible, in the process of preparing the histological slides, attention was paid on the obtainment of section representative for the entire lymph node. Nevertheless, analysis of a higher number of patients could possibly lead to a statistically significant difference in the number of GCs of inflamed GCs compared to uninflamed GCs within mesenteric lymph nodes.

In the establishment of microbial flow cytometry, difficulties developed in the IgG measurement, as the antibody staining was performed adequately, but did not show differences. This led to the necessity of a positive control for the used antibody. Once this has successfully been performed, the experimental set up was further changed, to be able to exclude a methodological problem. Hence, checking the rate of detected events per second as a previous step before further staining was applied. This guaranteed, that not too many bacteria were used for the antibody staining and further analysis with the flow cytometer. Moreover, additional washing steps after serum incubation were introduced, as leftover serum components could possibly inhibit the binding of the antibody to anti-commensal IgG. After all these changes in the protocol, an IgG signal was eventually detected.

The following measurement of serum samples of IBD patients (n=15) and healthy controls (n=4) revealed, that the bacterial staining delivered reproducible results. Furthermore, analysis showed no significant difference in the relative frequency of bound IgG and IgA commensal bacteria compared to the healthy control group. However, as we have not performed adequate sample size calculations, a higher number of patient samples may change statistical results.

Nevertheless, the findings of microbial flow cytometry are at least showing a trend in supporting previous studies, indicating that a higher number of commensal bacteria can break through the damaged intestinal barrier of the inflamed tissue and thus trigger an immune response that leads to the production of IgG antibodies directed against them. In the experiments, the serum of a UC patient, who exhibited acute intestinal inflammation at the time of blood collection, was also tested and revealed a higher percentage of IgG^+ bacteria, than other tested serum samples. This result is consistent with Castro-Dopico et al.´s (2019) work, that showed, that UC patients have a higher amount of IgG-bound commensal bacteria, which subsequently further exacerbates intestinal inflammation.

One of the limiting factors regarding the methodology was that initially the stool sample, used for the preparation of the commensal bacteria, originated not from a healthy patient. Hence, the possibility arises, that this caused a higher percentage of both IgG^+ and IgA^+ commensal bacteria, as there could have already been antibodies bound to them before serum incubation, as a result of the damaged intestinal barrier. Additionally, the findings were based on a lower number of healthy controls (n=4) in comparison with the samples of IBD patients (n=15). Therefore, the investigation needs to be repeated with a more representative pooled stool

sample from healthy patients, and serum from IBD patients and healthy controls at the same time.

Thus, in terms of future experiments, it would be useful, to extend current findings in testing all serum samples of the diseased group and the healthy control group again in one day, to ensure preparation under the same conditions. It would be of tremendous importance to expand the healthy control group with additional samples for analysis. Moreover, all serum samples should be incubated with the same stool sample. In this way, more reliable and comparable results could be obtained, as there are fewer limiting factors. Furthermore, in order to receive a cleaner peak with less background by FACS analysis, the used sera should be heat inactivated, to block the complement system. To identify naturally occurring secretion of IgG and IgA antibodies to the gut, a separate sample should be prepared alongside without serum incubation.

As the results from this thesis are already showing a trend that further supports the hypothesis and the protocol for microbial flow cytometry delivers reproducible data, more work should be done, to be able to clarify the question, whether there is an increase in the relative frequency of bound IgA and IgG commensals in Crohn´s disease patients.

In this way, these findings provide a step towards a better understanding of the antibody responses in Crohn´s disease and call for further investigations to clarify the pathway and effect of IgG antibodies directed against commensal bacteria in CD, which at some point could eventually lead to a new therapeutic target.

6. Summary

In Crohn's disease, an inflammatory bowel disease, continuous inflammation of the gut leads to a damaged intestinal barrier. As a result, more commensal bacteria can break through this barrier and sustain or aggravate the inflammatory response. This chronic and unphysiological exposure of the adaptive immune system to commensal bacteria can lead to a pathological B cell receptor (BCR) class switch in B cells from Immunoglobulin A (IgA), which naturally occurs at mucosal surfaces, to Immunoglobulin G (IgG), which naturally results in the development of plasma cells and thus, formation of soluble antibodies towards commensals. Moreover, whether IgG binding affects disease severity, and potentially sustains inflammation, is insufficiently understood. As B cells are the precursors to plasma cells, and receptor rearrangement results in antigen-specific immunoglobulins, further characterization of this population can provide valuable information in the initiation of the pathological antibody response. In brief, after activation of the naïve B cell through an antigen specific to their BCR, B cells maturate and differentiate in germinal centers, which are usually located in secondary lymphatic tissue. Within this thesis, the aim was to further characterise potential changes in size and quantity of germinal centers in mesenteric lymph nodes draining diseased intestinal segments of patients with Crohn´s disease, and compare them to germinal centers from lymph nodes draining unaffected intestinal segments by immunohistochemistry. For the quantification and measurement of these germinal centers within mesenteric lymph nodes, we utilized purified anti-human CD19 antibody (BD Pharmigen) staining. 10 slides of each healthy and diseased paraffin-embedded mesenteric lymph nodes of Crohn's disease patients were stained and statistically analysed. Using this approach, we could demonstrate that germinal centers within inflamed mesenteric lymph nodes are not increased in number, but in size compared to uninflamed lymph nodes. Furthermore, in order to identify a possible difference in the relative frequency of bound IgG and IgA on commensal bacteria, microbial flow cytometry has been established and tested with samples of patients with IBD (n=15) and healthy controls (n=4). As no significant results from flow cytometry were obtained, due to various limiting factor, especially the low number of healthy controls, further experiments are necessary.

Overall, the project has, and is going to, tackle the question of antibody responses in Crohn´s disease and will contribute to a better understanding of this inflammatory bowel disease.

7. Zusammenfassung

Bei Morbus Crohn, einer chronisch entzündlichen Darmerkrankung, führt die chronische Entzündung des Darms zu einer Schädigung der Darmbarriere. Infolgedessen können mehr kommensale Bakterien diese Barriere durchdringen und die Entzündungsreaktion aufrechterhalten oder verstärken. Diese chronische und unphysiologische Exposition des adaptiven Immunsystems gegenüber kommensalen Bakterien kann zu einem pathologischen Wechsel der B-Zell-Rezeptorklasse (BCR) in den B-Zellen von Immunglobulin A (IgA), das natürlicherweise an den Schleimhautoberflächen vorkommt, zu Immunglobulin G (IgG) führen, was wiederum die Bildung von Plasmazellen und damit die Sezernierung von Antikörpern gegen Kommensalen zur Folge hat. Darüber hinaus ist nur unzureichend geklärt, ob die IgG-Bindung den Schweregrad der Erkrankung beeinflusst und die Entzündung möglicherweise aufrechterhält. Da B-Zellen die Vorläufer der Plasmazellen sind und eine Rezeptorumlagerung zu antigenspezifischen Immunglobulinen führt, kann eine weitere Charakterisierung dieser Population wertvolle Informationen über die Auslösung der pathologischen Antikörperreaktion liefern. Kurz gesagt, nach der Aktivierung der naiven B-Zelle durch ein für ihren BCR spezifisches Antigen reifen die B-Zellen aus und differenzieren sich in Keimzentren, die sich in der Regel im sekundären lymphatischen Gewebe befinden. Im Rahmen dieser Arbeit wurden mögliche Veränderungen in Größe und Anzahl der Keimzentren von entzündeten und gesunden mesenterialen Lymphknoten von Morbus Crohn Patienten mittels Immunhistochemie näher charakterisiert. Für die Quantifizierung und Messung dieser Keimzentren in den mesenterialen Lymphknoten verwendeten wir eine Färbung mit einem anti-humanen CD19-Antikörper (BD Pharmigen). Jeweils 10 Schnitte gesunder und entzündeter paraffineingebetteter mesenterialer Lymphknoten von Morbus Crohn Patienten wurden angefärbt und statistisch ausgewertet. Mit diesem Ansatz konnten wir zeigen, dass die Keimzentren in entzündeten mesenterialen Lymphknoten nicht in ihrer Anzahl, sondern in ihrer Größe im Vergleich zu nicht entzündeten Lymphknoten erhöht sind. Um einen möglichen Unterschied in der relativen Häufigkeit von gebundenem IgG und IgA auf kommensalen Bakterien zu identifizieren, wurde außerdem eine durchflusszytometrische Analyse etabliert und mit Proben von Patienten mit chronisch entzündlicher Darmerkrankung (n=15) und gesunden Kontrollpersonen (n=4) getestet. Da die Durchflusszytometrie möglicherweise aufgrund verschiedener einschränkender Faktoren, vor allem die geringe Anzahl an gesunden Kotrollpersonen, keine signifikanten Ergebnisse lieferte, sind weitere Experimente erforderlich. Zusammenfassend hat sich das Projekt mit Antikörperreaktionen bei Morbus Crohn befasst

und versucht zu einem besseren Verständnis dieser entzündlichen Darmerkrankung beizutragen.

8. Abbreviations

IBD	Inflammatory bowel disease
UC	Ulcerative colitis
CD	Crohn´s disease
BCR	B cell receptor
Ig	Immunoglobulin
IgA	Immunoglobulin A
IgG	Immunoglobulin G
GC	Germinal center
SHM	Somatic hypermutation
CSR	Class-switch recombination
HHC	Healthy household control
FcγR	Fc-gamma receptor
IHC	Immunohistochemistry
PBS-T	1x PBS containing 0.1% Tween 20
PBMC	Peripheral blood mononuclear cell
FACS	Fluorescence-activated cell scanning
BSA	Bovine Serum Albumin
FCS	Fetal Calf Serum
HPF	High Power Field
FSC	Forward scatter
SSC	Side scatter

9. References

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10. Figures

11. Tables