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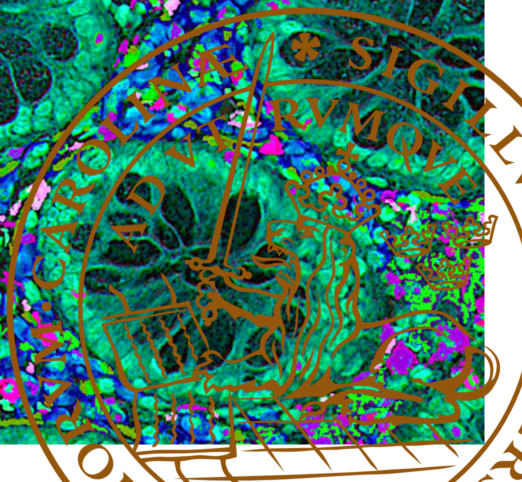
Intestinal immune cells in health and disease

Dissecting histological characteristics of inflammatory bowel disease and inflamed lung using quantitative immunohistochemical image analysis

MANAR ALYAMANI
EXPERIMENTAL MEDICAL SCIENCE | FACULTY OF MEDICINE | LUND UNIVERSITY



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Intestinal immune cells in health and disease

Dissecting histological characteristics of inflammatory bowel disease and inflamed lung using quantitative immunohistochemical image analysis

Manar Alyamani

DOCTORAL DISSERTATION

LUND UNIVERSITY

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine at Lund University to be publicly defended on the 1st of September at 13.00 in Sagerfalksalen, Department of Experimental Medical Science, BMC, Lund

Faculty opponent
Associate Professor Maria Magnusson
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Abstract:

The study of immunohistopathology can provide content and spatial information. We have developed a quantitative and computational approach to study the healthy and inflamed immune cell microenvironment in human and mouse tissues. The purpose is to uncover new knowledge about mucosal immunology that in turn will facilitate improvement of patient care.

In the first paper (I), we performed a broad immunological characterization of a knockout (KO) mouse with a focus on the intestinal immune system. The gene knocked-out was NPP7 which is an intestinal enzyme metabolizing dietary sphingomyelin. We collected and processed various tissues, and then performed quantitative immunohistochemical image analysis to characterize the immunological phenotype of the KO. In summary, we found significant effects of the KO on primarily intestinal and mesenteric lymph node T-cell subpopulations and dendritic cells, whereas other immune cell types were not affected.

In the second paper (II), we aimed at generating a new histopathological index to quantify the degree of inflammatory disease activity in patients with Inflammatory Bowel Disease (IBD). we quantitatively examined various immune cell populations in the intestinal epithelium and lamina propria, respectively, identifying populations that were significantly increased in intestinal mucosal biopsies from IBD patients as compared to control subjects. Selected parameters were put together to generate a new index, which could separate tissues from healthy controls and IBD patients with inactive disease from tissues collected from IBD patients with active disease to varying degrees.

In the third paper (III), we set out to dissect the heterogeneity of IBD, by applying advanced quantitative multiplex immunohistochemical image analyses for examining intestinal mucosal tissues regarding primarily the composition of an array of immune cell types and stromal cell types, in patients with IBD and in control subjects. The current manuscript describes the first step which is a rather coarse mapping, although still very complex and ambitious. This project aims at dissecting and decoding this heterogeneity which would be extremely useful for both IBD research and the treatment of patients with IBD.

The fourth paper (IV) examined autopsy lung tissues from 18 deceased COVID-19 patients by applying quantitative multiplex immunohistochemical image analysis and transcriptomics. This paper dissects the immense heterogenity in fatal COVID-19.

Key words: IBD, CD, UC, NPP7,S1P, IHC, mIHC, quantitative image analysis, COVID-19

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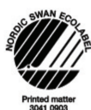

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To my family

لدواؤك فيكَ وما تـُبصـِـرُ.. ودواؤك منـــكَ وما تشــعرُ
وتـَحسب أنك جِرم صغير.. وفيكَ انطوى العالمُ الأكبرُ

الإمام علي بن أبي طالب كرم الله وجهه

The cure is in you and yet you don’t see it...
And the disease is from you, and you don’t feel it...

You deem yourself an insignificant thing...
And yet, in you the whole cosmos exists…

Imam Ali Bin Abi Talib

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Papers in this thesis

Paper I

Alkaline sphingomyelinase (NPP7) impacts the homeostasis of intestinal T lymphocyte populations. Manar Alyamani, Mohammad Kadivar, Jonas S Erjefält, Bengt Johansson-Lindblom, Rui-Dong Duan, Åke Nilsson, and Jan Marsal.

Frontiers in Immunology 2023 Jan 13:1050625

Paper II

Inflammatory bowel disease activity measured by quantitative immuno-histochemical image analysis of innate immune cells. Mohammad Kadivar, Manar Alyamani, Michiko Mori, Maryam Kadivar, Jimmie Jönsson, Erik Hertervig, Olof Grip, Lena Svensson, Jonas S Erjefält, and Jan Marsal.

Manuscript

Paper III

Dissecting the heterogeneity of inflammatory bowel disease applying quantitative multiplex immunohistochemical image analysis. Manar Alyamani, Caroline Sandén, Rana Bokhary, Viktoria Bergqvist, Mohammad Kadivar, Mohammed Binsalman, Jonas S Erjefält, and Jan Marsal.

Manuscript

Paper IV

Diffuse alveolar damage patterns reflect the immunological and molecular heterogeneity in fatal COVID-19. J.S. Erjefält, N. Costa, J. Jönsson, O. Cozzolino, K. Dantas, C. Clausson, P. Siddhuraj, C. Lindö, Manar Alyamani, S. Lombardi, A. Mendroni, L. Antonangelo, C. Faria, A. Duarte-Neto, R. Monteiro, J. Pinho, M. Gomes-Gouvêa, R. Pereira, J. Monteiro, J. Setubal, E. de Oliveira, J. Filho, C. Sanden, J. Orengo, M. Sleeman, L. da Silva, P. Saldiva, M. Dolhnikoff, and T. Mauad.

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Papers/projects not included in this thesis

V.
Lung Mast Cells Have a High Constitutive Expression of Carboxypeptidase A3 mRNA That Is Independent from Granule-Stored CPA3. Premkumar Siddhuraj, Carl-Magnus Clausson, Caroline Sanden, Manar Alyamani, Mohammad Kadivar, Jan Marsal, Joanna Wallengren, Leif Bjermer, and Jonas S Erjefält.
Cells 2021 Feb 3;10(2):309

VI.
Dynamically upregulated mast cell CPA3 patterns in chronic obstructive pulmonary disease and idiopathic pulmonary fibrosis. Premkumar Siddhuraj, Jimmie Jönsson, Manar Alyamani, Pavan Prabhala, Mattias Magnusson, Sandra Lindstedt, and Jonas S Erjefält.
Frontiers in Immunology 2022 Aug 2; 13:924244

VII.
Ki-67 as a disease activity marker in Ulcerative Colitis and Crohn's Disease. Manar Alyamani, Arvid Zetterquist, Rana Bokhary, Viktoria Bergqvist, Mohammad Kadivar, Mohammed Binsalman, Jonas S Erjefält, and Jan Marsal.
Manuscript.

VIII.
Comparative analyses of the Ulcerative Colitis Histopathologic Indices Geboes score, Nancy Index, and Robarts Histopathology Index. Manar Alyamani, Rana Bokhary, Viktoria Bergqvist, Mohammad Kadivar, Mohammed Binsalman, and Jan Marsal.
Manuscript.

Abbreviations

Alk-SMase	Alkaline sphingomyelinase
AMLC	Additive multiplex labelling cytochemistry
AMP	Antimicrobial peptide
AP	Alkaline phosphatase
APC	Antigen presenting cell
ARDS	Acute respiratory distress syndrome
CD	Crohn’s disease
CD c	Crohn’s disease colon
CD i	Crohn’s disease ileum
COVID-19	Coronavirus disease 2019
CRP	C-reactive protein
CT	Computerized tomography
Ctr	Control
Ctr c	Control colon
Ctr i	Control ileum
DAB	Diaminobenzidine
DAD	Diffuse alveolar damage
DC	Dendritic cell
DEG	Differentially expressed gene
DSS	Dextran sulphate sodium
ECP	Eosinophil cationic protein
ELISA	Enzyme-linked immunosorbent assay
FAE	Follicle associated epithelium
FFPE	Formalin fixed paraffin embedded
GHAS	Global histologic disease activity score
GI	Gastrointestinal
GPCR	G protein-coupled receptor
GS	Geboes score
Hb	Hemoglobin
HBI	Harvey–Bradshaw index
HD	Human defensin
HIER	Heat induced epitope retrieval
HRP	Horseradish peroxidase

HT	Heterozygote
IBD	Inflammatory bowel disease
IE	Intraepithelial
IEC	Intestinal epithelial cells
IFNγ	Interferon gamma
Ig	Immunoglobulin
IgA	Immunoglobulin A
IL1RL1	Interleukin-1 receptor-like 1
KO	Knockout
LI	Large intestine
LP	Lamina propria
LT	Lymphoid follicular tissue
MC	Mast cell
MLN	Mesenteric lymph node
MPO	Myeloperoxidase
MRI	Magnetic resonance imaging
MRP	Myeloid-related protein
NETs	Neutrophil extracellular traps
NK	Natural killer cell
NOD2	Nucleotide oligomerization domain 2
NPP7	Nucleotide pyrophosphatase/phosphodiesterase 7
NSAID	Nonsteroidal anti-inflammatory drugs
PAF	Platelet activating factor
PDPN	Podoplanin
PG	Prostaglandin
pIgR	Polymeric Ig receptor
PP	Peyer's patch
RELMβ	Resistin-like molecule beta
ROI	Region of interest
RORγt	Retinoic acid receptor-related orphan nuclear receptor gamma t
ROS	Reactive oxygen species
S1P	Sphingosine-1-phosphate
S1PR	Sphingosine-1-phosphate receptor
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SCFA	Short-chain fatty acid
SED	Subepithelial dome
SES-CD	Simple endoscopic score for Crohn's disease

sGS	Simplified Geboes score
SI	Small intestine
sIgA	Secretory IgA
SIRPα	Signal regulatory protein α
sm	Smooth muscle
SM	Sphingomyelin
TCM	Central memory T-cells
TCR	T-cell receptor
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
Treg	Regulatory T-cell
UC	Ulcerative colitis
UCEIS	Ulcerative colitis endoscopic index of severity
VEGF	Vascular endothelial growth factor
WBC	White blood cell count
WFDC2	Whey acidic protein four-disulfide core domain 2
WT	Wildtype
XCR1	X-C motif chemokine receptor 1

Overview of intestinal anatomy and histology

The intestine is a vital part of the digestive system, and its main function is absorption of nutrients from ingested food in addition to eliminating waste. The intestine is also an entrance point for many bacterial and viral pathogens, as well as a reservoir of a vast and diversified microbial community that is recognized to have a profound impact on both host physiology and pathophysiology.

The intestine is divided into two main sections: the small intestine (SI) and the large intestine (LI). Each section has its distinct anatomical and histological features (figure1-2).

The small and large intestines

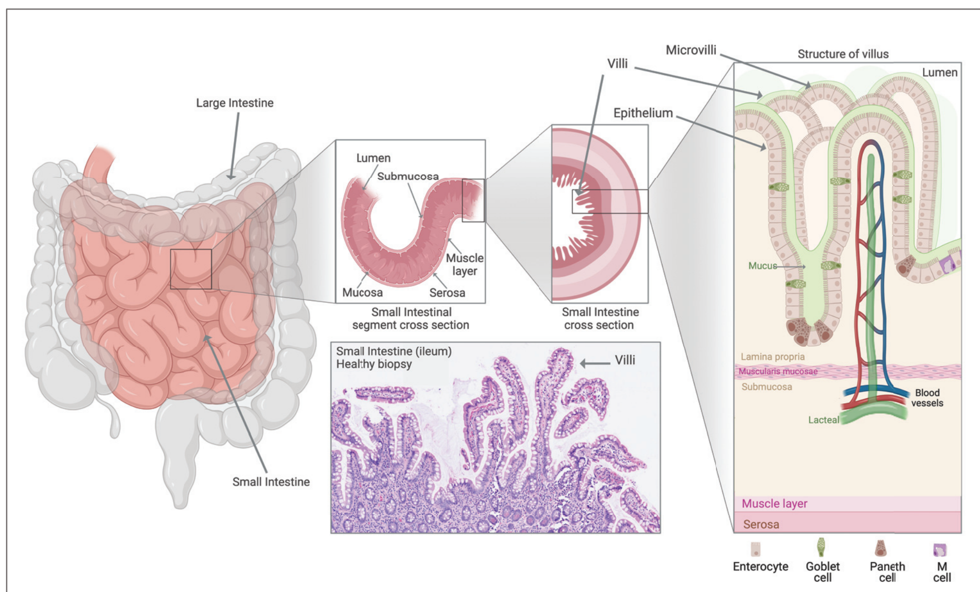

Figure 1. Small intestinal anatomy and histological features.
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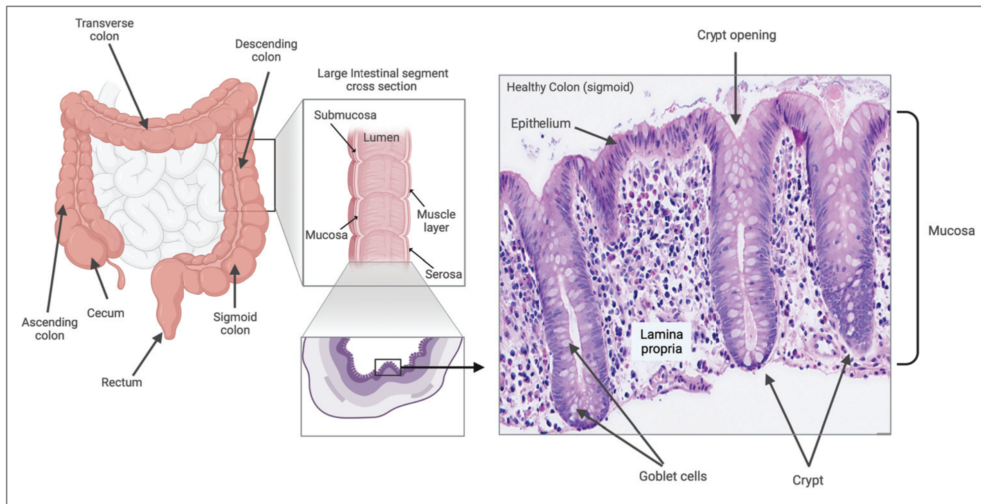

Figure 2. Large intestinal anatomy and histological features.
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Anatomy and physiological Function

The small intestine (SI) is a long, narrow, and coiled tube that stretches from the pyloric sphincter to the ileocecal valve. It is approximately 6 to 7 meters long, highly convoluted, and is positioned in the central and lower parts of the abdominal cavity. The SI is characterized by finger-like extensions called villi that extend into the lumen and increases the absorptive and digestively active epithelial surface area. It is divided into three parts; the duodenum, jejunum, and ileum.[1] On the other hand, the large intestine (LI) is shorter (~1.5m) and wider in diameter. The LI starts at the caecum, followed by the ascending colon, the transverse colon, the descending colon, the sigmoid colon, and the rectum, terminating at the anus.[2] Unlike the SI, the surface of the LI is flat, and villi are absent, but there is an enormous number of glandular crypts.

The main functions of the SI are digesting food and absorbing nutrients and water and moving food along the gastrointestinal (GI) tract. The first part called the duodenum is responsible for enzymatic (chemical) digestion. The jejunum, the second part, churns food back and forth with the enzymatic juices for mechanical digestion. The ileum is the last as well as the longest part of the SI. It is thinner and narrower with few blood vessels, and food spends more time here. The ileocecal valve separates the ileum from the colon.[2,3] In contrast to the SI, the LI does not produce enzymes, and does not digest food, but it absorbs water and electrolytes.[4]

Histological features

The GI tract from the esophagus to the rectum has four major layers: a lining mucosa, a submucosa, a muscularis, and an outermost adventitia or serosa.

The mucosa

The mucosa is the innermost layer and varies along the GI tract according to regions but always consists of a single-layered epithelium lining a lamina propria of loose connective tissue with the muscularis mucosae beneath. Intestinal glandular crypts of Lieberkühn are found between the small intestinal villi (figure.1). One of the major differences between the SI and LI, is that the LI lacks villi. Instead, the surface of the LI is relatively flat, limiting potential damage caused by solid stools transiting the LI. The surface is also punctuated by invaginations of intestinal glands (crypts of Lieberkühn) commonly known as the colonic crypts that resemble thick-walled test tubes with a central opening towards the intestinal lumen (figure.2) and are primarily lined by the goblet cells. Lymphoid follicles (see below for further details) are scattered throughout the entire mucosa. [5,6]

The epithelium

The epithelium is the first barrier against luminal content and lies on a thin collagenous basement membrane. It consists of a single layer of specialized cells with diverse functions. [7]

The four major cell types of the intestinal epithelial cells (IECs) are i) absorptive **enterocytes**, ii) mucin producing **goblet cells**, iii) hormone releasing **neuroendocrine cells** (also called enteroendocrine cells), and iv) anti-microbial **Paneth cells**. They develop from continually dividing stem cells that are located at the crypt base.[8] The IECs undergo differentiation and maturation as they migrate along the crypt upwards to the villus tips, which takes 5-6 days. They are then sloughed into the lumen, except Paneth cells that last longer (>30 days) and migrate downwards to the base of the crypt after differentiating from stem cells.[5,6,9]

The crypt epithelium is mainly covered by absorptive enterocytes. However, it also contains Paneth cells which are pyramidal cells located within the crypt, and increasing numbers are seen from the duodenum towards the ileum in the SI, but they are rare in the colon. Goblet cells also populate the crypts and decrease in numbers towards the villus tip, while increasing in frequency towards the distal colon. The composition of mucin from goblet cells differs from the mucin from the absorptive enterocytes. Neuroendocrine cells are found primarily at the base of

the crypts and the cell-population of the LI is generally less diverse than in the SI.[5,6,10] The predominant IEC type of the villi in the SI is the absorptive enterocyte.

The lamina propria

The intestinal lamina propria is a tissue layer composed of numerous structures and cell-types, including collagen, elastic fibers, blood vessels, lymphatics, fibroblasts, myofibroblasts, and various innate and adaptive immune cells. It is located beneath the epithelium, making up an important part of the mucosa of the intestine. It plays a crucial role in supporting the epithelial layer, maintaining the structural integrity of the intestine, and participating in various immune functions that will be detailed further below. The lamina propria also houses lacteals which are specialized lymphatic capillaries responsible for absorbing dietary fats.

The muscularis mucosae

The muscularis mucosae separates the lamina propria from the submucosa. It is the deepest layer of the mucosa, lying close to the submucosa. It is composed of several thin layers of smooth muscle fibers oriented in various directions, and is thin, from 3 to 10 cells thick. A distinction of the colonic muscularis mucosae is that it is thicker, and the thickness increases from the cecum to the anal canal.[5,6]

Overview of the Intestinal immune system in healthy state

The dynamic and everchanging environment in the intestinal lumen poses a challenge to the intestinal immune system. It is perhaps not surprising, then, that the intestine has the most diverse and highest number of immune cells in the body. The immune system of the intestine can be principally divided into inductive sites and effector sites (figure 3).

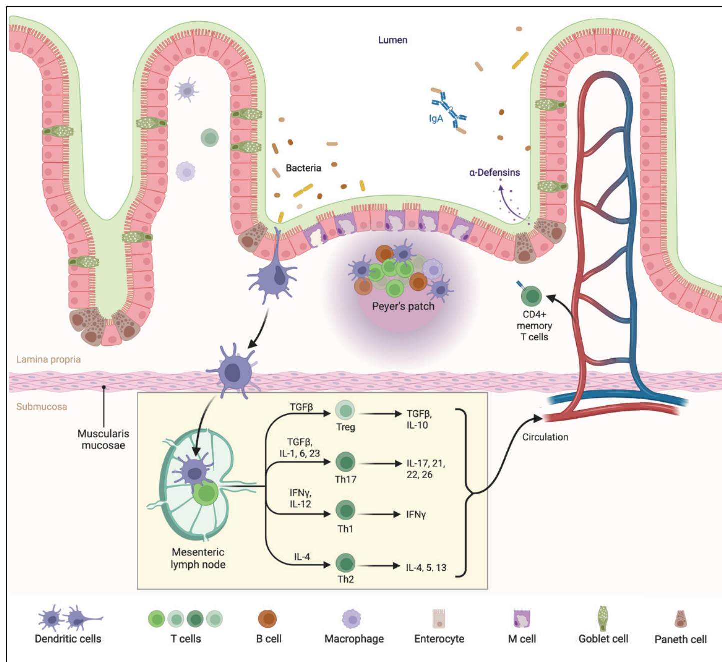

Figure 3. The intestinal mucosal immune system
The intestinal mucosal immune system consists of three main compartments: the epithelium, the LP, and the GALT. DCs and macrophages can extend protrusions into the epithelium to sample luminal antigens before migrating to the draining mesenteric lymph nodes (MLN) to activate naive T-cells. M-cells are specialized epithelial cells that are located in the epithelium covering Peyer's patches (follicle associated epithelium, FAE) delivering antigens to DCs, macrophages, and other APCs in the subepithelial dome (SED). After being activated by APCs, naive T cells in secondary lymphoid tissues proliferate, upregulate gut-homing surface molecules, exit the lymph node (directed by S1P) through efferent lymphatics to the blood circulation, and finally home back to the intestinal LP or the epithelium.*Figure created by BioRender.com*

Intestinal inductive sites

Inductive sites are where antigens sampled from mucosal surfaces induce naïve cognate T and B lymphocytes. The inductive sites include organized mucosa-associated lymphoid tissue (MALT) and local/regional mucosa-draining lymph nodes (LNs). In the intestine the corresponding sites are gut associated lymphoid tissue (GALT) and mesenteric lymph nodes MLN, respectively.

Gut associated lymph tissue

Gut associated lymph tissues (GALT) are organized tissue structures, that consist of subepithelial lymphoid aggregates occupying the mucosa or submucosa. They are characterized by a top layer named the follicle-associated epithelium (FAE), which contains differentiated intestinal epithelial cells known as microfold cells (M cells). These take up and transport antigens from the lumen through the subepithelial dome (SED) region, that is rich with dendritic cells (DC). DCs then can present those antigens to adaptive immune cells[11]. M cells also allow entry for several intestinal pathogens. [12] The best characterized GALT structures are **the appendix** and the **Peyer's patches (PP).** The PP develop before birth, are located in the antimesenteric side of the small intestine and are enlarged and increased in number in the distal part of the ileum. In humans, PP comprise hundreds of B cell lymphoid follicles with surrounding, relatively small, T cell areas. **Solitary isolated lymph tissues (SILTs)** are another type of GALT structures. They develop after birth and have variable size, and maturation stages ranging from small **cryptopatches** to mature **isolated lymphoid follicles (ILFs)** and can be found throughout the small and large intestines. SILTs cannot be seen macroscopically, unlike the PP, and they have only a single lymphoid follicle. In addition, the organization into a B cell follicle and a T cell area is less clear. Similar to PP, they have germinal centre reactions, indicating active humoral activation. **The cecal patches** around the ileocecal valve, **the colonic patches** throughout the colon, and the rectum are all parts of GALT.[13–16]

Mesenteric lymph nodes

The mesenteric lymph nodes (MLNs) are encapsulated secondary lymphoid organs structured as typical lymph nodes including components such as a cortex, a paracortex, and a medulla, as well as lymphatic sinuses and blood vessels. MLNs are located in the mesentery, where they are arranged in clusters along the length of the mesentery, accompanying the blood vessels supplying the intestines. Studies have shown that different parts of the intestine are drained to different specific nodes among the MLNs.[17,18] The MLNs receive lymphatic drainage from the intestinal mucosa including drainage through lymphatic vessels from the

intestinal villi and the lamina propria that converge to form larger vessels that transport lymph fluid containing immune cells, antigens, and other substances to the MLNs. Naïve lymphocytes that encounter their cognate antigen in the MLNs may get activated, proliferate, differentiate and subsequently home as effector or regulatory cells back to the intestinal mucosa. The homing route follows efferent lymphatics from the MLNs through the thoracic duct to the blood circulation, and from there to the intestinal mucosa through a diapedesis process.[19,20]

One of the important functions of the MLN and GALT is aiding with tolerance to commensal bacteria and food antigens by inducing local and systemic immunological tolerance, known as oral tolerance. This occurs under homeostatic conditions when conventional dendritic cells (DCs) produce IL-10 and transforming growth factor-β (TGF-β), stimulating the differentiation of tolerogenic regulatory T cells. A break in this process may lead to a hyperaggressive immune response that in turn may lead to diseases such as IBD and celiac disease.[21–23]

The MLNs function as a fire wall between the intestinal lumen and the systemic immune system and circulation. In the healthy state, MLNs prevent commensal bacteria as well as antigen charged DCs from spreading systemically where they likely would trigger strong proinflammatory immune responses.[23]

Mechanisms of T-cell differentiation and migration at inductive sites

T cell differentiation in the healthy intestine starts with the uptake of antigens which may take place through multiple routes, one of them being the specialized M-cells that can transfer microbes and microbial antigens through the mucosal epithelium from the lumen to the subepithelial dome in Peyer's patches.[24] Another route is used by soluble antigens that can diffuse through epithelial tight junctions and taken up by subepithelially located antigen presenting cells. Antigens can also be directly sampled by transepithelial dendrites protruding from dendritic cells or CX3CR1^{+} macrophages in the lamina propria to the intestinal lumen.[25] Antigen-charged DCs upregulate their expression of chemokine receptor C-C motif receptor 7 (CCR7) which directs their migration to the inductive site which offers a meeting point for DCs from the LP and I lymphocytes that have entered into gut-draining lymph nodes and PP from the peripheral blood.[26] DC-mediated T-cell activation demands three signals: 1) T-cell receptor (TCR) binding of antigenic peptides presented by the major histocompatibility complex class II (MHCII); 2) Co-stimulation by CD28-CD80/CD86 binding; and 3) Cytokine signalling mostly delivered by the antigen presenting cell (APC). Once T cells are activated, they upregulate the expression of CD69, CD25 (IL-2 receptor-alpha chain), CD44, and CD45RO, and downregulate the expression of CD62L, CCR7, and CD45RA; and start secreting the cytokine IL-2 which mediates autocrine stimulation of proliferation. After a proliferation and differentiation period of 3-4 days[27] the T-cells leave the inductive site (mediated by a sphingosine-1-phosphate (S1P)

gradient) and traffic to their effector site (LP). The homing process to the intestinal mucosa is mediated by various homing molecules including $\alpha_4\beta_7$ integrin and the chemokine receptor CCR9.[19,20,28]

A subset of T-cells differentiates into long-lived memory cells, re-expresses CD62L and CCR7 which enables them to recirculate centrally as opposed to in the periphery and are thus called central memory T-cells. The remaining CD62L^{neg} CD45RO CD44^{+} T cells will move to the gastrointestinal lamina propria, where they reside as memory CD4^{+} T-cells. T cell homing to the gut requires the expression of integrin α4β7, which interacts with endothelial cells in blood vessels that express mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1). The epithelial cells of the small intestine produce CCL25 which binds to CCR9, a chemokine receptor expressed on lymphocytes homing to the intestinal mucosa. Regardless of T-cell phenotype or function, signals from the APC and MLN milieu influence the regionalization of the mucosal imprinted T-cell response.[29–31]

Intestinal effector sites

The effector sites are where primed and differentiated adaptive immune cells exert their effector function. In the intestine, these sites are the intestinal lamina propria and the overlying epithelium.

The intestinal epithelium

The intestinal epithelium is vital for preserving gut homeostasis and it acts both as a physical barrier and as an organizing center for immune defense and crosstalk between bacteria and immune cells. In order to protect the vast surfaces that are in contact with the outer world, the epithelial surface is covered by mucus produced largely by **goblet cells** that increase going down the GI tract. The colon has a thicker layer of mucus organized in two layers with the denser one attached to the epithelial cells. Production of mucus has been shown to be regulated by immune mediators, such as leukotrienes, interferon-γ (IFNγ), IL-9 and IL-13.[32–34] The mucus also has antimicrobial functions, it forms a gel that forms a physical barrier, in addition to being constituted of mucin glycoproteins that are toxic to many bacteria. The looser mucins produced in the small intestine also provides a medium to which antibodies and antimicrobial peptides can cling to. Flaws in mucus synthesis have been shown to cause increased infiltration of commensal bacteria into the colonic epithelium.[35] This in turn is thought to facilitate the development of conditions such as IBD and colon cancer.[36,37]

Intestinal epithelial cells (IECs) have been shown to express pattern recognition receptors (PRR), i.e., pathogen associated molecular patterns (PAMPs) and

damage associated molecular patterns (DAMPs), which recognize components from microbial pathogens and host cell components released during cell damage or cell death, respectively. When the IECs get activated through the PRRs, they produce mediators that recruit and activate cells of the immune system.[38] The expression of various PRRs may vary along different regions of the intestine. Some examples of PRRs expressed by colonic IECs are TLR2, TLR4, and CD14.[39,40]

Paneth cells contain secretory granules that are rich in various host protective proteins and peptides collectively known as antimicrobial peptides (AMP), such as α-defensin[41], lysozyme, secretory phospholipase A2, LPS-binding protein. In addition, Paneth cells may produce proinflammatory mediators including TNFα, IL-1β and IL-17A.[42] Regenerating islet-derived protein IIIγ (REGIIIγ) is another mediator that Paneth cells have the capacity to produce, in response to IL-22. TLR stimulation, nucleotide-binding oligomerization domain 2 (NOD2) stimulation, or cholinergic nerve signals.[43,44] In addition to having antimicrobial functions, Paneth cells are crucial for intestinal crypt homeostasis.[45] Indeed, defects in Paneth cell function have been suggested to lead to the development of Crohn's disease.[46]

Neuroendocrine cells are found dispersed throughout the intestinal crypts and villi. They are divided into several types according to their major secreted hormone.[47] They also act as chemosensors, detecting harmful substances, resulting in protective responses.[48] Gut microbiota produces various metabolites that can potentially modulate neuroendocrine cells, generating hormonal signals that reflect epithelial integrity and microbial composition.[47]

Intraepithelial lymphocytes (IEL) are a considerable and diverse population of lymphoid cells that are localized in between the IECs often abutting the basement membrane, promoting epithelial integrity. They are mostly TCR^+ and include both γδ and αβ T-cell subsets. They enter the epithelium by two alternate routes; either after antigen encounter in peripheral lymphoid tissues and these have been named induced IELs, or immediately after their thymic development and these have been named natural IELs.[49] It is estimated that there is approximately 1 IEL per 10 IECs in the small intestine with a lower number in the colon.[50] IELs usually express activation markers, i.e. CD69 and CD44, and do not recirculate.[51] They also express other specific surface receptors such as the chemokine receptor CCR9, which interacts with CCL25 that is produced by IECs. This receptor-ligand pair is involved in the recruitment of IELs to the gut mucosa. In addition, intestinal IELs express integrin αEβ7 (αE is also known as CD103), which in turn interacts with E-cadherin expressed on the basolateral side of IECs, to enable entry and retention of IELs in the intestinal epithelium.[50,52] The majority of IELs also express a the homodimer CD8αα, the function of which has not been fully elucidated but has been suggested to play a role in repressing TCR signaling.[53] Induced IELs include conventional $CD8\alpha\beta^+TCR\alpha\beta^+$ and $CD4^+$ T-cells which may be stimulated upon epithelial entry to express CD8αα. Natural IELs on the other hand have been

described to express CD8αα early on, together with either the αβ or γδTCR.[50,53,54] This paradigm however was adjusted and updated by data from our group showing that there is an additional subset of IELs that are $CD8\alpha\beta^{+}TCR\gamma\delta^{+}$.[55] Interestingly, these novel $CD8\alpha\beta^{+}TCR\gamma\delta^{+}$.[55] Were shown to be decreased in numbers in active IBD.[55] IELs are the first line of immune defense in the gut, at the same time as their homeostasis is dependent on microbial signals.[56] The main function of IELs is thought to be to maintain intestinal integrity, having both cytotoxic and regulatory functions, producing perforin, granzymes, and IFN-γ.[56,57]

The intestinal lamina propria

Intestinal immune cells are mainly found in the GALT structures. However, a large amount and wide range of immune cells are also present in the lamina propria (LP) as well as interspersed between the epithelial cells as mentioned above. The LP contains both innate and adaptive immune cells, including macrophages, dendritic cells, mast cells, T-cells, B-cells and innate lymphoid cells (ILC).[52] The epithelium however mostly contains T-cells of the unconventional type, and thus these two compartments are immunologically very different. Together, the intestinal LP and epithelium comprise the largest population of T-cells, plasma cells and macrophages in the body.

Adaptive immunity of the lamina propria

The **lamina propria (LP) T-cell** population is comprised of both $CD4^{+}$ T-cells and $CD8^{+}$ T-cells, with an estimated ratio of 2:1. LP T-cells are believed to differentiate from conventional naïve T-cells that get activated in secondary lymphoid tissues and traffic to the intestinal LP, and they exhibit an effector memory phenotype. The $CD4^{+}$ T-cell subpopulations are highly diverse within the intestines.[58,59] The predominant subpopulations in the LP are regulatory T-cells (T_{regs}), IFNγ producing Th1 cells, and IL-17 producing Th17 cells.[60]

FoxP3 T_{regs} produce the anti-inflammatory cytokines IL-10 and TGFβ. They are influenced by local secretion of TGFβ from the epithelium, neighbouring phagocytes, and stromal cells, in combination with luminal production of short chain fatty acids (SCFAs) and retinoic acid (RA), which all contribute to the differentiation of T_{regs} that supress exaggerated abnormal immune responses to commensal bacteria and food antigens.[61,62] Th1 cell differentiation involves the cytokine IL-12, and the signal transducer/transcription factors STAT1, STAT4, and T-bet.[63] The role of LP Th1 cells is to control intracellular pathogenic infections including viruses and bacteria, but they have also been implicated as the effectors in several autoimmune and immune-mediated diseases.[64–66] The cytokines IFNγ and TNFα, produced by Th1 cells, activate innate immune cells

including neutrophils, macrophages, and structural tissue cells such as myofibroblasts and epithelial cells.[67,68] Th2 cell differentiation requires IL-4 and the signal transducer/transcription factors STAT6 and GATA3. Th2 cells control helminth infections and extracellular microorganisms, and mediate allergic reactions and asthma.[69] Naïve $CD4^+$ T cells differentiate to Th17 cells in response to IL-6, IL-23, and TGF-β.[70–72] IL-6 signals via STAT-3, inducing the expression of the transcription factor retinoic acid receptor-related orphan nuclear receptor gamma (RORγt) leading to the production of IL-17 and IL-22.[73,74] IL-17, produced by Th17 cells, is believed to play a role in autoimmune and chronic inflammatory diseases. In addition, Th17 cells collaborate with T_{reg} to promote the repair of epithelial barrier in colitis.[75–77]

γδ T-cells can be found in the LP but reside primarily in the epithelium, where they can comprise up to 40% of the IELs in the intestinal mucosa. However, they only make up a minor part of the lymphocytes that circulate in the blood and peripheral organs. They are regarded as crucial cells in the first line of defence against infections and in the healing of intestinal wounds. γδ IELs display plasticity, immunoregulatory qualities, and absence of MHC restriction. Their value as a potential therapeutic target is yet unknown, although there is mounting experimental and clinical evidence of their involvement in CD and other forms of inflammatory bowel disease (IBD).[55,78]

B cells in the lamina propria are scarce and found mainly in GALT structures.[79] Effector cells from the B lineage are antibody producing plasma blasts and plasma cells that are abundant in the LP.[79] The majority of **plasma cells** in the gut produce Ig M and IgA, while systemic plasma cells primarily produce IgG.[80] IgA is produced either as a monomer or a dimer, with the latter being the most common at mucosal surfaces and named secretory IgA (sIgA). The IgA dimer, linked by a J-chain, diffuses across the basement membrane, and is then bound to the polymeric Ig receptor (pIgR) on the basolateral surface of the IECs, transcytosed to the apical side where the pIgR is cleaved with the secretory component remaining associated with the IgA dimer, protecting it from proteolytic cleavage. Pentameric IgM is secreted by a similar mechanism. In the lumen, IgA binds to antigens and microbes, and prevents access or attachment to the intestinal epithelium and neutralizes microbial toxins, contributing to intestinal homeostasis and barrier integrity.[81–83] However, intestinal IgA can also enable the transport of luminal antigens to APCs in GALT.

Innate immunity of the intestinal mucosa

Innate immune cells, including macrophages and DCs, as well as non-immune cells, including epithelial cells and myofibroblasts, sense microbial antigens through pattern recognition receptors (PRRs) that detect pathogen associated molecular patterns (PAMPs). As mentioned previously, Toll-like receptors (TLRs) and intracytoplasmic receptors, including nucleotide-binding oligomerization

domain (NOD) like receptors (NLRs), are examples of PRRs. An efficient innate response against pathogens is ensured by PRR signalling cascades that activate nuclear factor (NF)κB which in turn activates genes coding for primarily pro-inflammatory mediators. The crosstalk between innate and adaptive immune responses is greatly aided by PRR signaling, which also encourages APC maturation as seen by an increase in co-stimulatory molecules necessary for effective antigen presentation and T-cell activation.

The healthy intestinal mucosa does not harbor **neutrophils**. However, usually the recruitment of neutrophils is the first wave of immune responses and is a sign of injury, infection, or inflammation in the mucosa.[84,85] It has also been reported that they can migrate into the epithelium, further enhancing inflammation.[86–88]

Macrophages are the most abundant innate immune cell in the healthy LP. They have a number of crucial roles in maintaining intestinal homeostasis, such as phagocytosis, the breakdown of dead tissue cells and pathogens, and the generation of mediators that promote epithelial cell renewal. They also release considerable amounts of IL-10, which not only inhibits inflammation by reducing pro-inflammatory responses to stimuli like TLR,[89–91] but also enhances the survival and function of local FoxP3^{+} T_{reg}s in the mucosa.[92,93] In addition, the ability of macrophages to respond to IL-10 themselves is of importance in maintaining homeostasis. Mice with IL-10 receptor signalling knocked out in macrophages displayed spontaneous colitis.[94,95] Moreover, macrophage production of IL-1β in response to microbiota has been suggested to be important for the induction and development of Th17 cells in the healthy state of the small intestine.[96]

Mucosal DCs serve as crucial immune system modulators. They represent a surveillance system in mucosal tissues, as well as expert APCs that promote the differentiation of naïve T and B-cells. DCs express high levels of CD11c and MHC-II and can be divided into numerous subsets with distinct functions based on their molecular expression. Historically, CD103 and CD11b were used to subdivide DCs, but in later years the field has focused on the markers X-C motif chemokine receptor 1 (XCR1) and signal regulatory protein α (SIRPα/CD172a) to define the subpopulations of classical DCs (cDCs). cDC1 are efficient in inducing cytotoxic CD8^{+} T-cells and Th1 responses and are XCR1^{+}SIRPα^{-}. cDC2 on the other hand primarily drive Th2, Th17, and T_{reg} responses, are XCR1^{-}SIRPα^{+}, and can be further subdivided into CD103^{+} or CD103^{-} subpopulations.[97,98] CD103^{+} DCs can generate regulatory CD4^{+}Foxp3^{+} T-cells by secreting retinoic acid (RA) and TGF-β when activating the naïve T-cell.[99–101] Importantly, RA also induces the expression of the gut-homing molecules α4β7 and CCR9 on both CD4^{+} and CD8^{+} T-cells as well as B-cells. Finally, CX3CR1^{+} DCs are able to sample and process antigens both from the circulation and lumen.[102]

After maturation in the bone marrow, **eosinophils** enter the circulation and migrate to the GI tract. Eosinophils are recruited to the intestine via the CCR3 ligand CCL11 (also known as eotaxin-1)[103–105] and by α4β7 integrin.[106-107] Intestinal eosinophils are present already during fetal life, and[102] have been suggested to play a role in tissue repair and barrier integrity both in the healthy state and under inflammation.[104,105,108] Furthermore, a large proportion of these eosinophils express the cytokine receptor interleukin-1 receptor-like 1 (IL1RL1/ST2) which is the receptor for IL-33 which has been shown to be upregulated in the inflamed intestinal mucosa.[109,110] In addition, eosinophils have been shown to have a role in IgA class switching in PP, in the generation and maintenance of IgA^+ plasma cells, T_{regs}, and $CD103^+$ DCs, contributing to intestinal immune homeostasis.[111]

Mast cells can be present anywhere along the healthy GI tract, primarily in the LP and the submucosa, while only a few are found in the epithelium.[112–114] Upon cell activation through IgE receptors, mast cells may release mediators such as histamine, leukotrienes, proteases, prostaglandins, and cytokines. Functions exerted include regulation of epithelial barrier integrity and permeability, and intestinal peristalsis. In addition, mast cells are part of important two-way interactions with the local nervous system.[115,116] It has also been reported that mast cells can vary functionally by location; in the small intestine they produce a protease that is TGF-β dependent[117] while in the colon they have a more proinflammatory phenotype.[118]

Basophils contribute to protective immunity, notably against parasitic diseases. They are few in numbers which makes it difficult to study their functions. Nevertheless, they have been implicated in allergies. Basophil elimination reduces the susceptibility to intestinal food allergy, demonstrating the critical importance of basophils in these conditions.[119]

Inflammatory bowel disease

Inflammatory bowel diseases (IBD) are chronic diseases with a relapsing and remitting course that do not result from specific pathogens. IBD comprises primarily Crohn’s disease, ulcerative colitis, and IBD-unclassified (IBD-U). Sometimes, depending on the type of categorization, microscopic colitis including collagenous colitis and lymphocytic colitis are included in the IBD group.

Crohn's disease (CD) can affect any part of the digestive tract, from the mouth to the anus in a non-continuous manner, but most frequently affects the small intestine and colon. The patchy or segmental pattern of inflammatory engagement has given rise to the term “skip lesions” which describes the inflamed part of the intestine, surrounded by healthy intestinal segments. CD is characterized by transmural inflammation which leads to complications such as fibrotic strictures, fistulas, and abscesses. Symptoms of CD include abdominal pain, diarrhea, weight loss, fatigue, and fever.[120] **Ulcerative colitis (UC)**, on the other hand is limited to the colon, causing more superficial inflammation with ulcers in the lining of the colon and spreads proximally from the rectum in a continuous manner. Symptoms of ulcerative colitis include bloody stools, diarrhea, urgency to have a bowel movement, and weight loss.[121] Both CD and UC typically require continuous treatment with medications, but sometimes also surgery. The diagnoses of CD and UC are based on a combination of endoscopic, histopathologic, clinical, and laboratory findings, and sometimes cross-sectional imaging.[122]

Although it has been found that there may be significant distinctions between CD and UC, such as differentially enriched immune-cell subpopulations[123] and genetic variants, such as NOD2 and PTPN22, that increase the risk of CD but may be protective against UC.[124] Another example is smoking which increases the risk and severity of CD, whereas it attenuates inflammation in UC. Early studies of familial clustering of cases and twin studies led to the recognition of a genetic component, with a somewhat stronger role in CD compared with UC.[125]

There is currently a lack of comprehensive understanding of the pathogenic mechanisms that underlie the various clinical presentations of IBD. Beyond the two main IBD subgroups, there is additional phenotypic subgroups; for instance, ileal and colonic CD seem to represent separate entities, and colonic CD can be further divided into subgroups based on gene expression profiles.[126]

Immunopathology of IBD

The pathogenesis of IBD involves a complex network of genetic, environmental, epithelial, microbial, and immunological factors. In the next section these will be discussed.

Genetic aberrations

There is increasing evidence to suggest that a central mechanism behind IBD is an inappropriate inflammatory response to commensal gut microbes in genetically vulnerable individuals. The important role of host-microbe interactions in the pathogenesis has been highlighted by genetic studies.[127–133] Among the most notable genetic discoveries are those describing polymorphisms in genes coding for the nucleotide oligomerization domain 2 (NOD2),[125,127,130] autophagy genes, and components of the IL-23/Th17 pathway.[125] NOD2 is an intracellular receptor binding bacterial peptidoglycans, and autophagy allows cells to control and eliminate a variety of intracellular materials, including pathogens.[129,134] The IL-23/Th17 pathway mediates pathogen defence but also pathological inflammation in IBD.[135,136] The autophagy gene ATG16L1 has been linked to CD but not to UC thus far.[134]

IBD and the intestinal microbiome

The microorganisms that inhabit the gut make up the intestinal microbiome. The intraluminal microbiota influences energy metabolism, provides important nutrients, and influences the development of the intestinal immune system.[137] The gut microbiota is formed at birth, but during the first years of life it undergoes changes. While the individual fecal microbiota of each adult is largely stable over time, changes do arise in response to environmental and developmental factors, illness, and other conditions.[138–140] Microbiome-host interactions are typically beneficial but can also become detrimental, as shown in both human studies and in murine colitis models. Experiments with antibiotic treatment have shown effects on clinical disease activity in humans and inflammatory levels in murine models.[141] Although a number of individual pathogens such as *Escherichia coli*[142] have been implicated in the pathogenesis of IBD, none have been proven to be causal as such; instead, it seems to be the microbial antigens found in the intestinal lumen that induce the pathogenic inflammation in IBD. Patients with CD or UC exhibit a depletion and reduced variety of members of the mucosa-associated phyla when compared to control subjects. Also, the Firmicutes to Bacteroidetes ratio has been shown to be decreased in both CD and UC.[143,144] It is not known if these changes in the microbiome, which collectively are called dysbiosis, is a

primary cause or if they represent a secondary effect of the inflammation and potentially medications.

A defective intestinal barrier

The intestinal epithelium is a highly dynamic single layer of epithelial cells connected by tight junctions with immune cells imbedded in between the IECs. The epithelium is the frontier towards the luminal contents and the microbiome, playing a major role in shaping the immune responses of the gut. The tight junctions between the epithelial cells are crucial to seal the space in between IECs preventing luminal content to access the lamina propria.[145] In IBD, these spaces are not effectively sealed which leads to a defective barrier.[145] Numerous pro-inflammatory cytokines, e.g. IFNγ and TNFα, have been shown to increase permeability and to prompt apoptosis of IECs.[146–148] This defect could be primary or a result from inflammation. Further protective mechanisms include the specialized IECs Paneth cells and goblet cells, which secrete anti-microbial peptides and protective mucus, respectively.

An important role of goblet cells in the healthy state[8,35,37,149] is to produce protective mucus consisting of MUC family proteins, antibacterial peptides such as trefoil factor (TFF), and resistin-like molecule beta (RELMβ), supporting epithelial repair, promoting mucosal healing and limiting luminal microbes.[150,151] Initial studies suggested that the mucus layer was diminished in UC due to fewer goblet cells.[152] More recent studies show that long-term goblet cell alterations could be caused by chronic inflammation in UC, resulting in abnormal mucus secretion, which in turn could be facilitating the recurrence of UC.[153] MUC2 is the mucin with the highest expression in the colon of both healthy individuals and UC patients.[154,155] However, colon biopsies from UC patients with acute inflammation have shown a mucus layer that is thinner and highly penetrable, while UC patient in remission displayed impenetrable mucus.[152,156,157] Whey acidic protein four-disulfide core domain 2 (WFDC2) is a protein secreted by colonic goblet cells, that is down-regulated in active ulcerative colitis. This may result in abnormalities in mucus layer formation, increased microbial colonization and invasion, and weakening of the epithelial barrier.[153]

Paneth cells are secretory cells found in the Lieberkühn crypts, next to the intestinal stem cells. The Paneth cells release AMPs and proteins, as well as other factors that aid in host defense and immunity. Defects in number, function and AMP release have been suggested to play a role in initiating inflammation in CD.[158,159] The CD-associated NOD2 polymorphisms have been linked to a decrease in Paneth cell-produced α-defensins and decreased antimicrobial defense.[160] Indeed, NOD2 has been shown to regulate the production of human defensin 5 and 6 (HD5, HD6).[161]

IEC destruction is characteristic of IBD, and both ileal and colonic epithelia exhibit increased cellular death in IBD.[162–164] This seems to be reduced by anti-TNF therapy, potentially accelerating mucosal repair in a subgroup of CD patients.[165] Although intestinal epithelial cell death being a physiological mechanism, the increased rate has been proposed to be related to the pathogenesis of IBD.[166] Given the importance of the many factors that maintain epithelial homeostasis and epithelial barrier integrity, subtle defects in epithelial components, IEC gene expression or function are likely to contribute to IBD pathogenesis.

Dysregulated immune responses

Aberrations in innate responses

In IBD, the epitheliuem together with the innate immune system make up the first line of defense. By interacting with PRRs expressed by innate immune cells, DAMPs and PAMPs stimulate the innate immune system, which comprises a number of cell-types, including neutrophils, monocytes, macrophages, and dendritic cells. Additionally, non-immune cells including myofibroblasts and IECs may detect and react to similar patterns and stimuli.

Neutrophils are short lived innate effector cells. Under homeostatic conditions they are not present in the intestine. They are abundant in the circulation and can be immediately recruited to sites of inflammation or infection.[167] When the intestinal barrier is damaged, neutrophils extravasate from the blood circulation to the site of inflammation, a process which is directed by a chemotactic gradient created by chemokines CCL8, CXCL10, and MIP-2). Other mediators including IL-1β, IL-6, TNFα, GM-CSF, G-CSF, and bacteria-derived chemicals also play a role in neutrophil recruitment. At the site of injury, neutrophils phagocyte and remove pathogens. Neutrophils degranulate, releasing reactive oxygen species (ROS) to the surroundings. Neutrophil activity is steeply increased in IBD.[168] Calprotectin, lactoferrin, and myeloperoxidase (MPO) are produced by neutrophils. Calprotectin is found in the cytosol of neutrophils and its levels in both feces and blood have been found to be increased in IBD patients. In addition, increased fecal calprotectin levels have been shown to predict relapses.[169] An additional function of neutrophils is the release of neutrophil extracellular traps NETs, which are mesh-like structures composed of DNA and its histone scaffold, as well as granule components such as MPO, neutrophil elastase, cathepsin G and protease 3. NETs extend from the active neutrophil membrane to confine extracellular bacteria and to activate complement proteins, which allows for contained bacterial lysis through their bactericidal effects.[170–173] The involvement of NETs in IBD has been described as double edged since it on the one hand hinders the spread of microorganisms [174,175] but on the other hand causes collateral

tissue damage and barrier impairment by releasing proteases to their surroundings.[176] Nevertheless, in addition to eliminating bacteria, neutrophils participate in wound healing and inflammation resolution by producing vascular endothelial growth factor (VEGF) which is a key mediator responsible for tissue remodeling and subepithelial fibrosis.[177]

Monocytes, a subset of circulating leukocytes, are recruited from the bone marrow to the blood, then they move to the gut where they are stimulated by cytokines and/or microbial molecules to differentiate into macrophages or dendritic cells.[178,179]

Tissue resident macrophages are highly plastic cells that are abundant in the intestinal mucosa; they are critical for the maintenance of a healthy mucosa. The primary function of macrophages is to remove and break down debris and pathogens, with little ability to stimulate naïve T cells, unlike dendritic cells that migrate to draining lymph nodes via CCR7 to activate naïve T cells.[26] One of the primary mechanisms that prevents macrophages from an inappropriate reaction to commensal bacteria is selective inertia to specific antigenic stimuli from the gut lumen.[180,181] When tolerance to luminal content breaks, conditions such as IBD arise.[182] Although having phagocytic and bactericidal potential, resident macrophages fail to release pro-inflammatory mediators in response to signals such as TLR ligands.[183] Instead, they carry out a variety of important homeostatic activities, such as assisting in the maintenance of T_{regs}, and supporting epithelial cell renewal through the production of IL-10 and prostaglandin (PG) E2.[183–185] In one study, the lack of responsiveness to anti-TNFα therapy in patients with IBD was linked to failure in the IL-10 signaling pathway, which is required for an anti-TNFα-induced shift towards a regulatory type of macrophages.[186–188] In some circumstances activated effector macrophages phagocytose microbiota and increase the production of TNFα, IL-6, IL-12, and IL-23, promoting IBD. Microenvironmental signals can cause macrophages to polarize into two distinct phenotypes; classically activated macrophages (M1) that have proinflammatory functions such as producing TNFα, IL-6, IL-12, and IL-23; or alternatively activated macrophages (M2) that contribute to healing of the mucosal barrier and maintaining homeostasis. The cellular polarization may in turn influence the progression of disease.[189,190]

DCs have been linked to IBD pathogenesis[191] through both genetics and function. They are crucial for regulating the outcome of the interaction between the immune system and the microbial contents of the gut. In human IBD as well as murine models of intestinal inflammation, activated DCs accumulate at areas of inflammation.[192–194] These cells display various activation markers and an enhanced TLR response, and are phenotypically different from the hyporesponsive DCs that serve to maintain mucosal homeostasis.[195–198] DCs in the healthy intestine exhibit modest levels of TLR2 or TLR4, while the expression levels of both of these TLRs increase on DCs during IBD.[199] MLN DCs are central in directing the

differentiation of helper T-cells to the various subtypes. T-cells isolated from MLN of CD patients showed an increased production of IL-17 and IFNγ which in turn was due to the increased secretion of IL-23 and decreased secretion of IL-10 from MLN DCs. Interestingly, this was not observed in UC patients or healthy controls.[200–202]

Mast cells (MC) contribute to intestinal homeostasis by modifying epithelial integrity.[203,204] Mediators produced by mast cells are increased in inflamed IBD tissue segments compared to those that are non-inflamed.[205] One study reported that CD patients with higher recurrence rates displayed three or more mast cells around inflamed nerve bundles (plexitis).[206,207] In vitro, activated MCs produce significant quantities of TNFα. Given the efficacy of anti-TNFα agents in the treatment of IBD, it is worth noting that one study showed that the majority of TNFα expressing cells in the LP were MCs.[208]

Other innate immune cells have also been described as part of the complex pathophysiology of IBD. Basophil numbers, for instance, are increased in the blood of patients with both CD and UC, and in mucosal samples from IBD patients basophils were shown to be accumulated.[209] Eosinophils have also been reported in several studies to be higher in number in IBD mucosal samples.[210–212] The role of eosinophils in IBD is unclear.[213] Cytotoxic granule proteins from eosinophils may cause damage to the intestinal tissues and enhance inflammation, and eosinophils have been associated with intestinal fibrosis, but eosinophils have also been suggested to be protective with regards to[212] barrier function, to modulate the massive neutrophil influx in IBD, and to promote mucosal healing in UC.[214–216]

Aberrations in adaptive response

An important characteristic of chronic intestinal inflammation is the infiltration of proinflammatory T-cells into the LP.[217] Newly diagnosed IBD patients showed a distinct composition of T-cell subsets in mucosal samples.[218] A higher percentage of $CD4^+$ T-cells, T_{regs}, and central memory T-cells (T_{CM}), with lower proportions of $CD8^+$ T-cells and $CD103^+$ T-cells, was demonstrated in inflamed tissues compared to healthy controls. Correspondingly, once inflammation resolved endoscopically, levels of T-cells were comparable with controls.[19,218] In contrast, other studies have reported that the LP and the epithelium of IBD patients contain normal quantities of $CD8^+$ and $CD4^+$ T-cells, but that the cells exhibited a phenotype of enhanced activation.[219,220,221] Normally, the balance between T_{regs} and proinflammatory subsets is tightly regulated.[222] T-cells mostly exhibit a regulatory phenotype in the healthy state, whereas in IBD, Th1, Th2, and Th17 responses dominate, albeit together with increased numbers of T_{regs} trying to moderate the inflammatory activity.[223] Th1 mediated responses were formerly thought to be the cause of CD. The discovery of the Th17 lineage prompted a re-evaluation of the immune based mechanisms behind CD and the conclusions from CD animal

model studies. Nevertheless, there is a strong Th1 signature in CD with biopsies from CD patients displaying increased IL-12 and IFNγ levels, and T-cells from the LP of CD patients expressing high expression of STAT4 and Tbet.[224–231] The elevated levels of IFNγ responses indeed suggest a pathogenic role in IBD, however, the anti-IFNγ antibody fontolizumab was not therapeutically efficacious in clinical trials. In contrast, the more recent therapeutic antibody ustekinumab that specifically binds to the p40 subunit shared by IL-12 and IL-23, has been successful in treating CD.[232] IL-12 and IL-23 released by activated APCs support the differentiation of naïve $CD4^+$ T cells into Th1 cells and Th17 cells, respectively.[233] Th17 cells are induced by IL-6 and TGFβ, and their expansion and survival is supported by IL-23. Th17 cells are characterized by the secretion of IL-17A, IL-17F, IL-21, and IL-22. IL-17 is strongly related to both CD and UC, in contrast to IFNγ that is more associated with CD.[234] Patients with active UC and CD have considerably more IL-17 secreting $CD4^+$ T-cells than patients in remission.[235] Additionally, T-cells in mucosal samples from IBD patients express the transcription factors RORγt, STAT3, and IRF4 as well as the surface markers CD161 and IL-23 receptor which are associated with Th17 cells.[236,237] Elevated fecal IL-17 levels have been detected in active CD, and serum IL-17 levels were also significantly higher in both CD and UC.[235,238] Interestingly, it is not readily apparent how IL-17 secreting $CD4^+$ T-cells contribute to the pathogenesis of CD and UC.[239] Studies exhibit unfavorable as well as protective functions of IL-17 in IBD. Somewhat unexpectedly, clinical trials with anti-IL-17 antibodies were not successful, and the treatment was even associated with aggravation of disease. Data showing that IL-17 is important for regulating tight junctions of IEC and thus maintaining the mucosal barrier function, for epithelial repair following injury by promoting proliferation of crypt stem cells, and for immunity against fungi, could potentially explain the mechanisms behind the results from the anti-IL-17 clinical trials.[240–242]

γδ T-cells have been implicated in IBD.[78] Several studies have investigated the involvement of γδ T-cells in IBD, and their roles appear to be complex and context dependent. Some studies have reported an increased frequency of γδ T-cells in the peripheral blood and intestinal mucosa of individuals with active IBD, suggesting their potential contribution to the inflammatory process. These cells can produce IFNγ and TNFα, which can further promote inflammation and tissue damage.[78] On the other hand, other studies have suggested a potential protective role for γδ T-cells in IBD, by means of IL-17 and IL-22 secretion, promoting tissue repair and barrier function. Indeed, there is data showing a significant decrease in the numbers of γδ T-cells in active IBD.[55] Additionally, γδ T-cells may exert regulatory functions by suppressing the activity of other immune cells, such as αβ T-cells.[243–245]

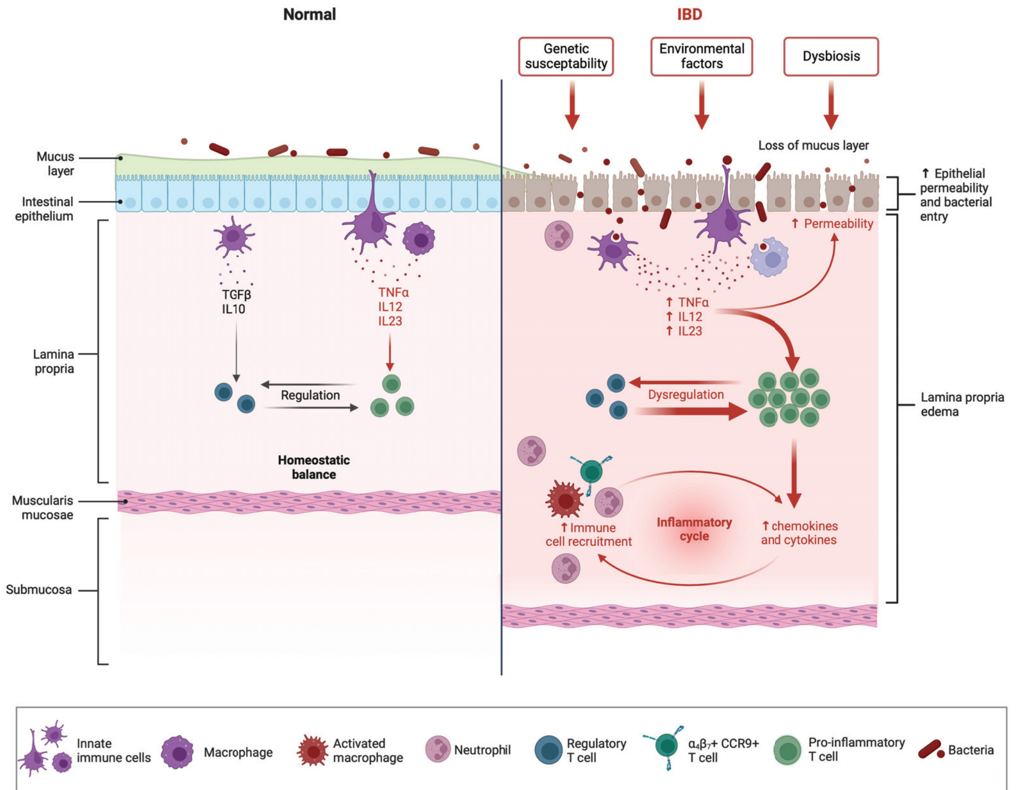

Figure 4. Immunopathology of IBD
IBD is thought to occur in genetically predisposed individuals by an inadequate immune response directed towards commensal bacteria of the gut. This aberrant immune response entails defects of both the innate and adaptive arms of the immune system. *Figure created by BioRender.com*

Clinical disease evaluation

Disease evaluation for patients with IBD involves assessing the activity and severity of the disease, monitoring its progression, and identifying potential complications. The evaluation typically includes a combination of clinical assessment, laboratory tests, endoscopic procedures, and cross-sectional imaging studies. Here are some key aspects of disease evaluation for IBD patients.

Clinical symptoms (clinical activity scores)

Healthcare providers evaluate the patient's symptoms and their impact on daily life. Common symptoms of IBD include abdominal pain, diarrhea, rectal bleeding, weight loss, and fatigue. These symptoms are evaluated based on frequency, duration, and severity.

The Crohn's disease activity index (CDAI) was developed as a single index of degree of illness for CD.[246] CDAI is based on clinical and biochemical variables that were derived from a week's diary.[246] Harvey and Bradshaw modified the CDAI to develop a simpler index, the Harvey-Bradshaw index (HBI) by employing only a single day's reading for diary entries based on clinical variables and it does not require biochemical testing. Each independent factor is coded in order that 0 denotes good health and increasing positive values represent greater severity of disease.[247,248] These variables include general well-being, abdominal pain, number of liquid/soft stools per day, abdominal mass, and extraintestinal manifestations. CDAI and HBI correlate well with each other. CDAI is essentially limited to clinical trials although it is considered the gold standard. The HBI on the other hand is used both in clinical trials and clinical routine patient management.

For UC, the Mayo score is commonly used in clinical trials to assess clinical disease activity.[249,250] The Mayo score is based on points given to each variable; frequency of bowel movement, rectal bleeding, physician's global assessment, and endoscopic findings. The partial Mayo excludes the endoscopic evaluation and correlates with the complete Mayo score.[249,251] In addition to the Mayo score, the Simple clinical colitis activity index (SCCAI) is also used to measure clinical activity in UC.[251] The SSCAI is determined by patients answers in a questionnaire regarding bowel frequency at day and night, urgency of defecation, blood in stool, general health and extracolonic manifestations.[251] The advantage of SCCAI is that is does not require the involvement of a healthcare provider.

Biomarkers

In IBD clinical routine practice, an endoscopic examination of the mucosa is the current gold standard for assessing disease severity. However, endoscopic examinations are costly, invasive, and rather burdensome for the patient. The optimal tool for monitoring of disease activity should ideally be accurate, practical, non-invasive, and cost-effective. Several serologic and fecal biomarkers including fecal calprotectin, fecal lactoferrin, serum C-reactive protein (CRP), and serum albumin are used to monitor the inflammatory activity in IBD.[252] These biomarkers however cannot stand alone in the evaluation of disease activity or in defining disease remission or relapse but can aid in the decision to move further with more cumbersome investigations, such as endoscopy or cross-sectional imaging.

Fecal calprotectin

Calprotectin is a calcium- and zinc-binding heterodimeric protein consisting of the subunits S100A8 and S100A9, has also been called calgranulin A/B and myeloid-related protein (MRP) 8/MRP14, and belongs to the S100 protein family.[253] It accounts for 60% of the cytosolic proteins in activated neutrophils, and its presence in stools is an indication of neutrophil presence in the intestinal mucosa. When the intestinal mucosa is injured due to an infection or inflammatory activity, mediators as calprotectin are released from neutrophils and activated macrophages to the surroundings, which together with whole neutrophils ending up in the intestinal lumen, leads to increased calprotectin levels in the feces.[254] In addition, it has been suggested that IECs under inflammatory conditions start expressing calprotectin, and when IECs are sloughed off into the lumen and degraded, this adds to the total amount of fecal calprotectin. Calprotectin is stable at room temperature for more than a week and is also relatively stable and resistant to enzymatic breakdown in feces and serves as a sensitive marker to detect gut inflammation. For isolated small intestinal inflammation, fecal calprotectin levels are lower and sometimes even false negative, whereas a severely inflamed rectum may generate extremely high values.[255] Moreover, increased fecal calprotectin levels are not specific to inflammatory activity in IBD, and elevated levels are also observed in colorectal cancer, GI infections, gastritis, and mucosal damage secondary to nonsteroidal anti-inflammatory drugs (NSAID) use. In addition to being a useful tool to detect intestinal inflammation, calprotectin is also biologically active and is involved in several processes, including cell differentiation, chemotaxis, tumorigenesis, apoptosis, immune regulation, antimicrobial activities by chelation of metal ions crucial for bacteria, and in activation of leukocytes, being a strong proinflammatory mediator.[256] Calprotectin can be measured using either a commercially available enzyme-linked immunosorbent assay (ELISA) or more recently developed faster methods which however are somewhat less accurate.[257]

C-reactive protein

C-reactive protein (CRP) is an acute-phase reactant. It is generated by hepatocytes in response to inflammatory cytokines such as IL-1β, IL-6, and TNFα and has a short half-life of about 19 hours. It is a commonly used serum indicator of systemic inflammation and is also used as a biomarker of IBD activity.[258–262] Elevated CRP helps in distinguishing mucosal active illness from dormant IBD.[263–265] CRP is not disease specific, and increased levels occur in both GI tract and non-GI tract inflammatory disorders, tissue damage, diabetes, cancers, and cardiovascular disease.[266–269] CRP assessment is limited in terms of IBD sensitivity; normal CRP levels can be found in patients with clinically active IBD as well as asymptomatic patients with mild tissue activity.[270,271] Although CRP levels correlate strongly with CD activity at the group level, and CRP levels being

more often increased in CD compared with UC, it cannot be used to differentiate between the two conditions.[272]

Endoscopy

Endoscopy plays a crucial role in the diagnosis, monitoring, and management of inflammatory bowel disease. Colonoscopy and/or flexible sigmoidoscopy allow direct visualization of the rectum, colon, and the terminal ileum providing valuable information about the extent, severity, and nature of inflammation. Biopsies can also be taken during endoscopy to get histopathological support for the diagnosis by assessing the presence of characteristic features of IBD. In addition, endoscopy plays a vital role in monitoring the progression and severity of IBD. The degree of inflammation observed during endoscopy can guide treatment decisions, including the choice of medication, escalation of therapy, or consideration of surgical treatment. Furthermore, endoscopy is used to evaluate the response to therapeutics in IBD patients. Repeat endoscopies may be performed to confirm disease remission or to identify subclinical inflammation that is present despite the patient not having any symptoms of disease activity. Patients with long-standing IBD, particularly ulcerative colitis, have an increased risk of developing dysplasia (pr-ecancerous changes) and colorectal cancer. Prescheduled surveillance colonoscopies with biopsies are recommended to detect dysplasia early and initiate appropriate management, including endoscopic resection or surgical intervention.

Indices are used to evaluate and quantify endoscopic activity. Traditionally the Mayo score has been used which consists of four degrees of disease activity: Remission, mild, moderate, or severe disease activity. However, the Mayo score suffers from poor interindividual agreement. The ulcerative colitis endoscopic index of severity (UCEIS) was developed fairly recently to offer an assessment of endoscopic severity in UC with higher resolution. It compasses three variables (vascular pattern, bleeding, and ulceration) which are scored with regards to the most severely affected segment.[273,274] Unfortunately, there are no validated severity levels indicating mild, moderate, and severe disease activity.[275]

In CD the indices commonly used in trials are the Crohn's disease endoscopic index of severity (CDEIS) and the less complex simple endoscopic score for Crohn's disease (SES-CD), which show good correlation.[275–278] CDEIS evaluates four variables: the presence of deep ulceration, superficial ulceration, the length of ulcerated mucosa, and the length of diseased mucosa in the ileum, right colon, transverse colon, left colon, and sigmoid colon. In the same colorectal locations evaluated by the CDEIS, the SES-CD evaluates the following parameters: ulcer size, extent of ulcerated surfaces, extent of inflamed surfaces, and stenosis.

Histopathological evaluation of disease activity in IBD

Mucosal healing is the objective of the treatment of CD and UC. It is defined by complete endoscopic and histologic remission.[279] The reason why mucosal healing is a treatment target is that it is associated with better clinical outcomes. Endoscopic remission has traditionally been thought of as a singular criterion in the description of mucosal healing, however increasing evidence reveals that it does not reflect the full picture in IBD. Truly, microscopic inflammation can continue even though endoscopically the mucosa has a healed appearance. More than 40% of patients with endoscopic signs of remission, had persistent neutrophil infiltration in the LP. Thus, histology is used to establish the complete remission of mucosal inflammation. However, it is a matter of debate whether endoscopic healing or mucosal healing should be the generally applied treatment target since it may in some cases be difficult to reach mucosal healing without very intensive treatment. Clinicians need to consider the risk/benefit ratio of intensified treatment given that increased treatment intensity comes with both more frequent and more severe adverse events.[280–284]

Histological characteristics of UC and CD

IBD has specific histological characteristics,[285] and those of UC involve an increased density of inflammatory cells infiltrating the LP, which includes eosinophils and plasma cells. Notably, an increase in plasma cells is typically found in the LP near the base of the crypts. This feature is indicative of UC and is known as basal plasmacytosis.[286] The quantity of eosinophils varies, but their presence (more than three at the base of the crypts) increases the likelihood of a diagnosis of UC.[287] The presence of neutrophils in the LP indicates an active phase of the disease. Even more active disease activity is characterized by glandular and surface epithelial lesions, such as crypt abscesses, cryptitis (neutrophils found intraepithelially), mucosal erosions, and ulcers.[286] An additional histological hallmark is architectural aberration, which can mean crypt distortion, dilatation, and branching. Reactive epithelial variations such as mucin depletion and Paneth cell metaplasia may develop and are common in both active and chronic disease.[288]

The histological assessment of CD is challenging since inflammation tends to be noncontinuous, patchy, transmural, and may reside in segments outside reach of the endoscope.[285,289] Nevertheless, epithelial injury and neutrophil infiltration into the LP and/or the epithelium mark an active phase of the disease.[290,291] Aphthoid ulcers and epithelial changes including irregular villous architecture are also detected in CD. Deep ulcers and fissures, transmural inflammation, and epithelioid granulomas are diagnostic hallmarks of Crohn's disease.[286]

Histological activity indices

Only a few of histological activity indices in ulcerative colitis are partially validated until 2015 which are [292,293]the Geboes score[294] and Riley score[295]. Then new validated indices were introduced as the Nancy index suggested in clinical practice due to its simplicity in application[296] and the Robarts index which is preferred for clinical trials[293]. Histological grading systems in UC are determined by the number, quality, and distribution of colonic biopsy samples collected, as well as the histological features combined. Histological features include erosion or ulceration, and the density and infiltration of LP neutrophil and basal plasmacytosis.[297–299]

For CD, several histological scoring systems have been developed. The Colonic and Ileal Global Histologic Disease Activity Scores (CGHAS and IGHAS) are the best known[300–302].Criteria such as epithelial damage, architectural changes, infiltration of mononuclear immune cells in LP, infiltration of polymorphonuclear immune cells in LP, infiltration of polymorphonuclear immune cells in epithelium, erosions and or ulcers and finally the presence of granulomas.[300–302] It is discouraged to use of these scoring systems in clinical practice because of the complexity and the lack of data linking histological disease activity with Crohn's disease outcomes. Instead, the existence of histological inflammation in an endoscopically quiescent condition should raise the alarm against de-escalation of therapy.[303]

Cross-sectional imaging

In UC, cross-sectional imaging techniques are supplementary to endoscopic assessment of disease activity. Resolution of radiological abnormalities in UC is not regarded as a therapeutic target in clinical practice.[304,305] Abdominal radiography for UC patients assesses the magnitude of fecal residue, colonic dilatation, and mucosal islands (areas of healthy mucosa among ulcerated tissue). This is only applied in the severe acute setting.[304] Computerized tomography (CT) imaging in UC aids in assessing potential complications.[304,306] Ultrasonography and magnetic resonance imaging (MRI) show good sensitivity for assessing UC activity and disease extension.

In contrast, cross-sectional imaging has an important role in evaluating disease activity in CD.[304] The most effective tool for determining the presence and severity of perianal fistulae and other significant complications of CD is MRI. For evaluating luminal disease activity and strictures in CD, MRI and ultrasonography are the best imaging techniques.[304] The application of capsule endoscopy has also proved highly useful for evaluating CD activity of the small bowel. Recently, a so called pancapsule has been introduced, which may examine both the small intestine and the colon during the same session.

The choice of imaging modality depends on various factors, including the clinical scenario, disease location, and the specific question that needs to be answered. Often, a combination of different imaging techniques is used to obtain a comprehensive assessment of IBD.

Alkaline sphingomyelinase (NPP7)

Background

Sphingolipids are one of the most significant types of eukaryotic lipids. The very first sphingolipids were identified in the brain in the late nineteenth century, naming them sphingosine after the Greek legendary creature, the Sphinx, in recognition of "the many enigmas which it presented to the inquirer".[307] Sphingolipids are central components of cell membranes. The basic structure of sphingolipids consists of three main components: 1) The sphingoid base, which is a long-chain amino alcohol with the most common sphingoid base being sphingosine; 2) A fatty acid that is attached to the sphingoid base via an amide bond, forming an amide-linked long-chain base; and 3) The headgroup, which is a polar component attached to the sphingoid base through a linkage. The nature of the headgroup controls the class of sphingolipid, with a common headgroup being phosphocholine as in sphingo-myelin (figure 5).[308]

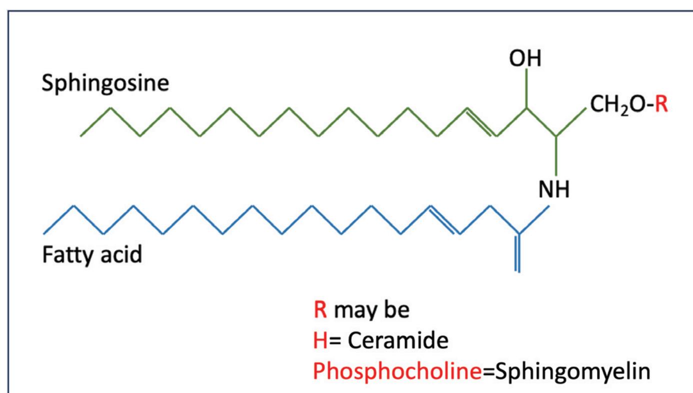

Figure 5. General structure of sphingolipids
The backbone of sphingolipids is made up of sphingosine that is linked to a fatty acid by an amide bond. Sphingolipids are categorized as ceramides or sphingomyelins depending on the type of residue in the side chain R.

Sphingomyelin (SM) is a type of sphingolipid, it colocalizes with cholesterol in the plasma membrane and in the membranes of Golgi and lysosomes.[309] Humans on a standard Western diet consume 0.3-0.4 g of sphingolipids daily, mainly derived from SM which is primarily found in meat, milk, egg, and seafood.[308] Nursing babies consume 150 mg of SM through milk per day. SM makes up

approximately 2% of the phospholipids in human bile.[309] In addition to dietary SM, the intestinal mucosal brush border is rich with sphingolipids, forming the endogenous source of SM.[309,310]

Hydrolysis of SM is an essential process with important biological implications. SM is digested and absorbed mainly in the small intestine where SM is metabolized by sphingomyelinases (SMases) and ceramidases. This group of enzymes has three known isoforms according to the optimal functional pH (acidic, neutral, and alkaline). In contrast to the widespread presence of acid and neutral SMases, alk-SMase (also called nucleotide pyrophosphatase/phosphodiesterase 7 [NPP7]) activity has been detected only in the intestinal mucosa, as well as in the liver. In the small intestine, NPP7 has hundreds of times the hydrolytic capability of acid and neutral SMases. NPP7 is an ectoenzyme found on the surface of the microvilli of IECs in the gut. Being located on the outside surface of the cell membrane, the enzymatically active site is accessible to the cell's external environment. The enzyme can also be released into the lumen by bile salt or pancreatic trypsin and enzymatic activity can be detected in both bile and feces.[311,312,313] NPP7 and neutral ceramidase are central enzymes of SM and ceramide metabolism in the GI-tract.[308] Ceramide, sphingosine, and sphingosine-1-phosphate (S1P), are produced after the absorption of sphingosine into enterocytes (figure 6).[314,315]

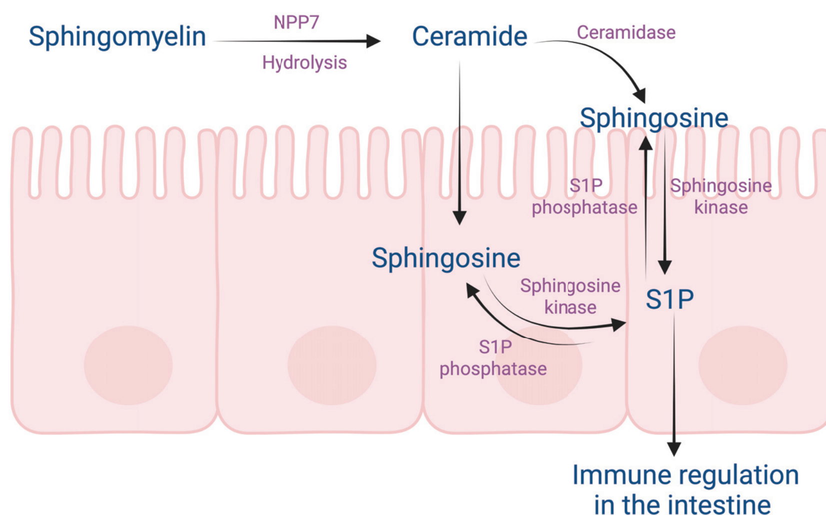

Figure 6. Sphingomyline hydrolysis by NPP7 at enterocytes

Alkaline sphingomyelinase and ceramidase on the apical membranes of epithelial cells break down dietary sphingomyelin into ceramide and then sphingosine. Ceramide is metabolized into sphingosine in epithelial cells. Sphingosine kinase converts sphingosine to S1P that subsequently participates in various immune functions. *Image created by BioRender.com*

NPP7 in intestinal immunity and IBD

NPP7 has multiple downstream metabolic pathways that result in the production of a variety of bioactive lipid messengers with various physiologic effects. Ceramide and sphingosine are lipid mediators that inhibit cell proliferation, induce apoptosis, and may counteract carcinogenesis.[316] In addition to producing ceramide, NPP7 can hydrolyze platelet activating factor (PAF) via phospholipase C activity, resulting in PAF deactivation. PAF is a proinflammatory factor that can be synthesized in a variety of tissues under inflammatory conditions,[317] and has been implicated in IBD and colorectal cancer pathogenesis.[318,319] It has previously been shown that NPP7 activity is downregulated during the early stages of colon cancer. In addition, reduced NPP7 activity has also been seen in patients with familial adenomatous polyposis and sporadic colon cancer,[320] but this impact on NPP7 is unlikely to be caused by mutations in the adenomatous polyposis coli gene.[321] Furthermore, Chen et al. found that the incidence of tumors in the colon is greater in NPP7 knockout (KO) mice than in wildtype (WT) mice.[322] In this study, the absence of NPP7 was also linked to greater tumor size, which was accompanied by reduced ceramide and increased S1P levels, as well as increased levels of β-catenin.[322]

Another downstream metabolite from SM hydrolysis by NPP7 is S1P, considered an important bioactive lipid mediator which regulates cell migration and proliferation. S1P is considered one of the more soluble sphingolipids and is found in low intracellular nanomolar concentrations, but in high concentrations in serum where it is coupled with lipoproteins and albumin.[323] S1P interacts with S1P receptors (S1PRs), which are high-affinity G protein-coupled receptors (GPCRs). There are five known S1PRs (S1PR1-5) that exhibit selective tissue expression.[324] S1P has been assigned several biological functions, including cell survival, cell migration, activation of innate immune cells, directing the egress of lymphocytes from lymph nodes and spleen, tumorigenesis, Th differentiation and fate switching, T-cell metabolism, supporting gut barrier function, neurodevelopment, fibrosis, endothelial cell barrier function, blood pressure, and cardiac rhythm.[325–331] The regulation immune cell trafficking, has been investigated as a new therapeutic target for a variety of immunological-mediated disorders such as multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus, and psoriasis.[332] S1P receptor modulators, such as fingolimod, ozanimod, etrasimod, and amiselimod are thought to exert their immunosuppressive function mainly by inhibiting the egress of lymphocytes from lymph nodes. However, S1P is found at increased levels locally in inflamed sites, including the inflamed gut, and it is conceivable that S1PR modulators have additional effects, locally at the site of inflammation.[333] The more selective S1P modulators (avoiding blocking of S1PR2-3 which has been associated with cardiac complications, and instead mainly targeting S1PR1) have been shown to be safe as well as efficient for

treating both CD and UC. These agents allow for both endoscopic and clinical improvement and remission.[334]

An earlier study, where NPP7 was administered rectally into the colon of rats with colitis once daily for one week, showed that NPP7 instillation led to lower colonic inflammatory scores and protection from inflammatory damage. In addition, TNFα expression was lower in the NPP7-treated group.[335] In another study, on human material, NPP7 levels were found to be decreased in chronic UC, indicating an anti-inflammatory effect of NPP7 in the colon.[336] In this context, it is interesting to note that some studies have suggested a protective role for S1P locally in the gut mucosa, supporting gut barrier function and homeostasis.[333,337,338]

Consequences of NPP7 deletion on the overall gut-related immune system in the homeostatic state has never been studied. The first publication examining immune cell populations in NPP7-related immunological compartments using quantitative image analysis of immune-stained tissue sections from homozygous and heterozygous NPP7 deficient mice, as well as wildtype mice, is included in this thesis. The findings revealed significant changes in T-lymphocyte populations in specific anatomical compartments, implying an important role for NPP7 in intestinal immune homeostasis.[339]

Mucosal immunology and COVID-19

Background

The immune systems of the various mucosal surfaces in the body have many common features and there is vivid crosstalk between these different compartments.[340] As a consequence, a whole research field dedicated to address the interconnections between the mucosal surfaces has emerged over the past few decades. In addition to the intestinal mucosa, which is the main focus of this thesis, the mucosal immune system includes the airways, genital tract, urinary tract, oral cavity, and the eyes. This connection between these various sites has been emphasized in multiple studies. For instance, disrupted IgA-microbiota interactions in the gut during infancy have been linked to an elevated risk for asthma in children, and the use of antibiotics in neonates has also been associated with a greater risk of developing asthma.[341,342] Furthermore, vaccine application in one mucosal site may generate protection in another mucosal organ, which was shown among other[343] pathogens for Entamoeba histolytica and herpes simplex virus-2 (HSV-2).[343,344] In addition, a viral infection may result in high levels of virus-specific immunoglobulins in different mucosal secretions, which was shown among other viruses for human immunodeficiency virus (HIV).[345] Dysbiosis in the gut microbiota has been associated with several types of lung disorders, including allergies, asthma, and cystic fibrosis.[346] Also, changes to gut microbiome caused by either diet, disease, or antibiotics have been shown to alter the immune responses and homeostasis in the airways.[347] Finally, there are several studies that have shown intestinal involvement in chronic obstructive pulmonary disease (COPD) as well as pulmonary manifestations of IBD.[348,349]

The immunological connection between the gut and lung is not surprising given that the sites share some similarities in terms of protective mechanisms and immune cell populations, but they also exhibit significant differences due to the distinct microenvironments and immune challenges they encounter.[350]The airways and the gut are both lined with mucosal surfaces and an epithelial layer, forming a physical barrier that prevents the entry of pathogens to the underlying tissues.[351] Both harbor commensal microbial communities that contribute to immune regulation and potentially could affect each other in a bidirectional fashion. Their

subepithelial secondary lymphoid tissues both lack afferent lymphatics and display specialized epithelial cells for antigen uptake. We are still learning about the complexities of the interactions and communications that occur between distinct mucosal compartments.[352] A broader approach to researching the mucosal immune system has been suggested to be taken for future improvements in mucosal immunology and development of new anti-inflammatory therapies and new vaccines.[240,340,352]

Interestingly, there have also been several studies showing effects from specific commensal gut bacteria as well as levels of short chain fatty acids (SCFA) derived from gut microbiota, on the susceptibility to COVID-19 as well as the disease course of COVID-19.[240,353,354] Furthermore, beneficial effects from specific diets, including Mediterranean diet, in the context of COVID-19 have been suggested.[240,353,354]There is also a connection between COVID-19 and the gut in the other direction, i.e. COVID-19 impacts the gastrointestinal tract in several ways, causing symptoms such as nausea, vomiting, diarrhea, abdominal pain, abdominal distension, hematochezia, and hematemesis.[355]The GI-tract can indeed become infected by the SARS-CoV-2 virus with virus replication taking place in the IECs.[356]This is made possible by the fact that small intestinal enterocytes (IECs) express the entry receptor angiotensin-converting enzyme 2 (ACE2).[357,358] The levels of ACE2 expression on the IECs are actually even higher than those found on the cells of the upper airways. In contrast, ACE2 expression is not found on intestinal goblet cells or intestinal immune cells.[356–358]

In late 2019, Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) began causing a pandemic of acute respiratory disease, named coronavirus disease 2019 (COVID-19). The clinical presentation of SARS-CoV-2 infection in humans varies, from being asymptomatic all the way up to serious respiratory failure. When SARS-CoV-2 binds to respiratory epithelial cells, it begins to replicate and migrates down the airways, and infects alveolar epithelial cells.[359,360] Since angiotensin-converting enzyme 2 (ACE2), which acts as a receptor for SARS-CoV-2 mediating virus entry into cells, is abundantly expressed on the apex of lung epithelial cells in the alveolar space, these cells display ample destruction in COVID-19.

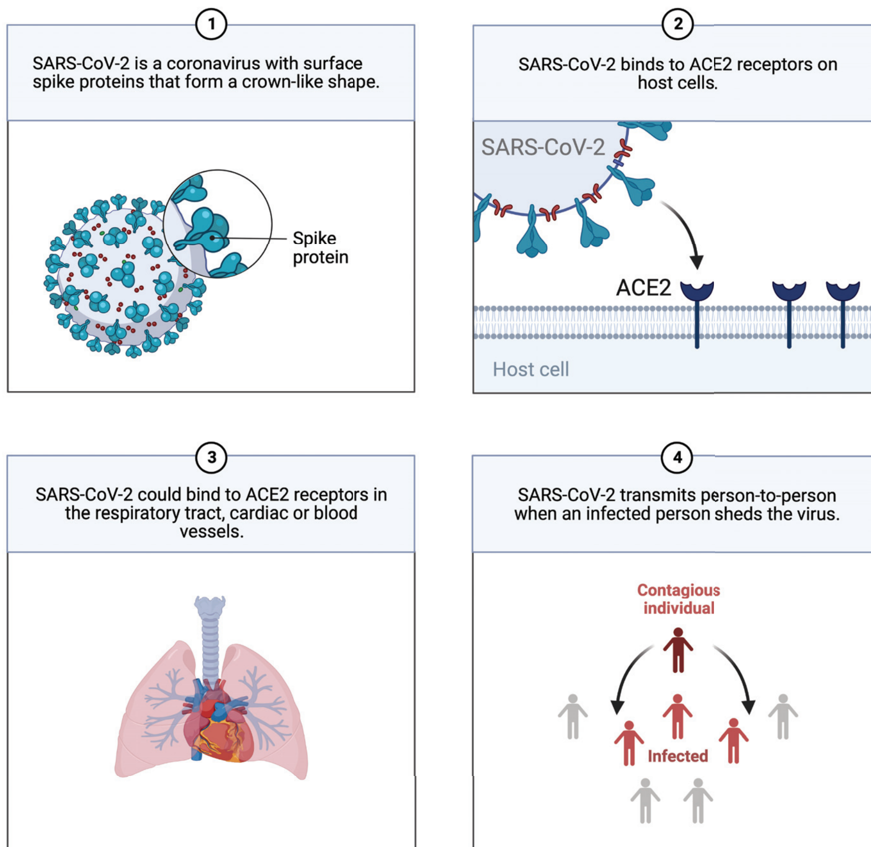

Figure 7. Key fetures of SARS-CoV-2
Panel 1 and 2) SARS-CoV-2 virus has spike proteins that attach to ACE2 which functions as a receptor, allowing for viral entry into cells. Panel 3) ACE2 receptors are distributed throughout the body, but the lungs and the heart are the mainly affected organs during SARS-CoV-2 infection. Panel 4) SARS-CoV-2 virus is highly contagious. *Figure created by BioRender.com*

The immune response to SARS-CoV-2

Direct infection of the airway epithelium is the first step in the immune response against SARS-CoV-2. After the initial infection, lung resident respiratory DCs acquire the invasive pathogen or antigens from the infected epithelial cells, become active, process the antigen, and move to the draining mediastinal or cervical lymph nodes.[361] Once in the lymph nodes, DCs present the processed

antigen in the form of an MHC/peptide complex to naïve circulating T-cells. When the peptide-MHC complex is bound by the T cell receptor (TCR) and co-stimulatory signals are delivered, the T-cells get activated, proliferate, differentiate, exit the lymph node and enter the blood circulation, and from there the activated T-cells home back to the site of infection in the lungs by means of trafficking molecules.[362] This lymphocyte activation and recruitment process is very similar to that described previously for CD and UC in the gut. $CD8^+$ T-cells mediate eradication of virus-infected cells while $CD4^+$ T-cells activate B-cells, promoting virus-specific antibody production.[363–365] The activated virus-specific effector T-cells release cytotoxic molecules including perforin and granzyme B, as well as antiviral cytokines like IFNγ, TNFα, and IL-2, and chemokines.[366] Increased plasma levels of immunological mediators, such as IL-6, TNFα, IL-10, granulocyte-colony stimulating factor (G-CSF), CCL2, CCL3, CCL7, CXCL9, and CXCL10 have been observed in patients with severe disease, reflecting the so called cytokine storm that takes place in the lungs.[366,367] Neutrophils and monocyte-macrophage lineage cells appear to be highly prominent and considerably expanded in quantity within COVID-19 lung tissue when compared to viral acute respiratory distress syndrome (ARDS) caused by influenza virus.[368]

Severe COVID-19 and its potential complications

The aggressive immune response seen in the lungs is driven by the rapid SARS-CoV-2 virus replication. The inflammation leads to epithelial cell death, denuded alveolar walls, alveolar haemorrhage and fibrin deposition with thick intra-alveolar hyaline membranes building up. ARDS and respiratory failure are the primary cause of death in COVID-19 patients.[369] The pathological picture of pulmonary changes in patients with fatal COVID-19 is defined as diffuse alveolar damage (DAD) of variable degrees and stages (acute, proliferative, and fibrotic).[370–372] Secondary events such as thrombosis, pulmonary and cardiac infarctions, and non-viral infections exacerbate the severe condition. Few studies have been conducted to date on the nature of the spatial cellular composition and relationships during the complex and heterogeneous alterations that occur in the lungs of patients with severe COVID-19.

Present investigation

Aim of the thesis

The overall aim of this thesis was to develop a new methodology to assess and quantify the inflammatory status in the human and murine intestinal mucosa, with regards to both quantitative and qualitative immunological aspects. This new methodology comprises histomic mapping of primarily immune cell patterns using computerized immunohistochemical image analysis.

Specific aims of the projects included in the thesis:

1. To investigate and broadly immunophenotype NPP7 knockout mice, using quantitative computerized immunohistochemical image analysis.

2. To develop a new histopathological index for quantification of the degree of inflammatory activity in patients with IBD, using quantitative computerized immunohistochemical image analysis.

3. To dissect the complex cellular composition, including various immune cell populations, of the intestinal mucosa in patients with IBD using multiplex immunohistochemical image analysis.

4. To decode the heterogeneity and immune complexity of COVID-19 using multiplex immunohistochemical image analysis.

The application of immunohistochemical image analysis in the investigation of the cellular and immunological microenvironment of the intestinal mucosa
- *A picture is worth a thousand words*

This chapter will briefly discuss the key steps for obtaining digital images from tissue samples, including sample preparation and the advanced immunohistochemical techniques applied in the projects of this thesis.

1. Sample preparation

Before the tissue is examined through a microscope or on the computer screen it needs to be processed properly to preserve correct morphology of various epitopes. The following are the main steps and procedures of the tissue preparation process: collection, fixation, embedding, sectioning, and staining.

i) Collection and fixation

Tissue sample collection is normally done through surgery or biopsy. In IBD, the samples are biopsies collected by special forceps during colonoscopies. There are a couple of different sizes of forceps, but using the standard size gives biopsies that are approximately 2x4 mm. After collection, tissue samples need to be fixed and the choice of optimal fixative is dependent on what type of analysis is to follow.

The commonly used fixatives are aldehyde, acetone, or alcohol. Aldehyde is an efficient cross-linking agent that is commonly used, and the most commonly used are formalin (10%, neutral buffered) or 4% paraformaldehyde. The aim of formalin fixation is to create chemical cross-linking of proteins within the tissue. This halts all cellular processes and locks cellular components in the location and orientation they were at the time of fixation, preventing degradation.

Acetone and alcohol are known as primary fixing solutions, that help to precipitate sugars and lipids. Acetone is frequently used for frozen tissue because of its penetrability and dehydration properties. In cryopreserved samples the tissue is frozen, sometimes after placement in a cryoprotective solution, and fixation is done after sectioning.

ii) Dehydration

Dehydration of tissues is an important step when paraffin is used for tissue preservation. Tissues contain water and when they are embedded in paraffin, water does not allow for adequate paraffin perfusion as water and paraffin are not miscible. Therefore, water in the tissue should be eliminated before embedding, a process called dehydration. Water is removed from the tissues by immersing them in first 70%, then 80%, followed by 90%, and finally in absolute alcohol. This stepwise approach prevents mechanical damage to fragile intracellular structures which may occur in case of rapid transfer of water from cells. Following dehydration, immersion in xylene eliminates any excess or leftover alcohol, preparing the tissue for embedding.

iii) Embedding

Tissue is treated and stored by embedding it in either paraffin wax or by freezing it in liquid nitrogen (cryopreservation). The most common method is to use formalin fixed paraffin embedded (FFPE) tissue blocks. FFPE tissues are ideal for storing tissue for research purposes but is also used in clinical practice. For some specific proteins and epitopes, such as some cytokines, chemokines, and enzymes, cryopreservation and acetone fixation is better for preserving the native structure and immunoreactivity, but for the vast majority of targets, FFPE is the best choice. Once processed, FFPE tissue is hardened and extremely stable. It does not require any specialized equipment for long-term storage and remains stable for decades with preserved morphological details. The method for each experiment should be chosen by considering factors such as antigen type, subcellular location, and intended method of detection.

iv) Sectioning

Paraffin embedded tissue is sliced to thin sections using a microtome. Recommended section thickness ranges from 3-6 µm. In our projects we used 3 or 4 µm thick sections. This can be compared to the diameters of immune cells: naïve T-cell 5-7 µm, activated T-cell around 10 µm, DCs 10-15 µm, and macrophages around 20 µm. After sectioning, the sections are mounted onto microscope glass slides. For long-term storage, FFPE sections can be kept at 4°C. With appropriate handling, the section preserves undisturbed tissue architecture and antigen structure, allowing for retrospective studies with high-quality immunohistochemical analyses.

v) Hematoxylin and eosin staining

Under the microscope, tissue samples are transparent or colorless. It would be difficult or impossible to identify structural features without contrast. Tissue sections must be stained to obtain contrast. A common approach of staining is to use a primary stain to emphasize specific targets of interest and a counterstain in a contrasting color to the primary stain. The secondary stain makes it possible to see the tissue morphology and pinpointing the location of the primary stain. The most frequently utilized stain in histopathology is a mixture of the chemical's hematoxylin and eosin (H&E). H&E is an all-purpose stain that is easy to use and dries quickly. This is the staining used in clinical routine practice, which most often is sufficient for skilled pathologists to diagnose or grade inflammation in tissue samples. Other more advanced staining techniques are primarily employed for identifying particular groups of cells and tissue components, as well as to see specific cellular activities. Immunohistochemistry and *in situ* hybridization are two of the most widespread advanced staining techniques.

2. Immunohistochemistry

Immunohistochemistry (IHC) is a microscopy-based technique for visualizing biological components in tissue samples, such as proteins or other macromolecules. The intuitive visual output of IHC indicates the existence and localization of the target protein in the context of various cell types, biological states, and/or subcellular localization within complex tissues. The IHC technique was developed in the 1940s.[373] It is consistently used in healthcare and pathology, for diagnostic purposes or to optimize patient treatment. IHC is additionally widely used in research to investigate the role of molecules of interest in both healthy and pathological cells and tissues at the molecular, cellular, or tissue level. There are multiple protocols available for various applications and tests that use

IHC or IHC-based approaches to visualize targets in tissues. Despite the fact that IHC is a reliable and well-established technique, novel assays or the detection of novel targets frequently require careful optimization depending on the tissue or the features of the target protein and/or reporter system.

The basic principles of immunohistochemistry

The basic steps in IHC are as follows: antigen retrieval, addition of primary antibody binding to the target antigen, applying a secondary antibody that binds the primary antibody, and application of a detection reagent to localise the primary antibody and thus the target antigen (figure 8).[374]

i) The initial step in IHC is usually antigen retrieval, which involves pre-treatment of the tissue to retrieve antigens hidden by the fixation step and making them accessible to antibody binding.[375] Antigen retrieval methods vary based on the target antigen and antibody, but most commonly require the chemical or physical breaking of protein crosslinks created by fixation, e.g. formalin. Physical treatments include heat and ultrasound, and chemical procedures include enzyme digestion and denaturant treatment. However, it is common to combine the two, for example denaturant treatment with heat. Heat induced epitope retrieval (HIER) is currently the most frequently used approach, with microwave ovens, pressure cookers, autoclaves, and water baths being the most common methods.[376]

ii) The second step is to add the primary antibody, which could either be monoclonal or polyclonal. The appropriate concentration of the antibody is titrated beforehand to optimize the contrast between positively stained tissue and nonspecific background staining.[377] In general, monoclonal antibodies that target a single epitope are more specific, whereas polyclonal antibodies that can bind many epitopes are more sensitive. Primary antibodies could be labelled and directly detected or be unlabelled and thus needing a secondary antibody for detection.

iii) Using reporter-labelled secondary antibodies for detection helps to increase sensitivity by means of signal amplification, as well as for making it easier to perform multiple staining on the same section. The selection of secondary antibody is dependent on what species the primary antibody's is and Ig isotype (class). There are numerous reporter-labels to choose from, including fluorescent compounds and enzymes (e.g. horseradish peroxidase [HRP] and alkaline phosphatase [AP]).

iv) The last step entails the application of a chromogenic substrate (e.g., diaminobenzidine [DAB]) which generates a colored stain after incubation.[374,378]

Background staining can be caused by nonspecific antibody binding, which is more common with polyclonal antibodies, and/or endogenous peroxidase activity which is more of a problem in tissues with a high concentration of hematopoietic

components, such as bone marrow. Nonspecific antibody binding can be reduced by incubating the tissue with normal serum from the same species as the secondary antibody or with a universal blocking agent (readily available) before the secondary antibody is applied. Endogenous enzyme activity can be reduced by pre-treating the tissue with hydrogen peroxide solutions before applying antibodies.[378,379]

Assessment of quality is essential, and every procedure should include both positive and negative controls. Positive controls are tissues known to contain the antigen that the specific antibody is directed to, and ought to be run on the same slide as the tissue of interest so that the control tissue is subjected to the same reaction conditions as the sample tissue. To control for nonspecific binding of the primary antibody, an isotype-matched negative primary antibody or, in the case of a polyclonal antibody normal serum from the same species, should be tested. To control for nonspecific binding from the secondary antibody, negative controls in the form of sample tissue stained as the experimental tissue minus the primary antibody. False positives and negatives can occur because of the immunohistochemistry process itself, as well as a variety of additional factors such as preparation and fixation.[379]

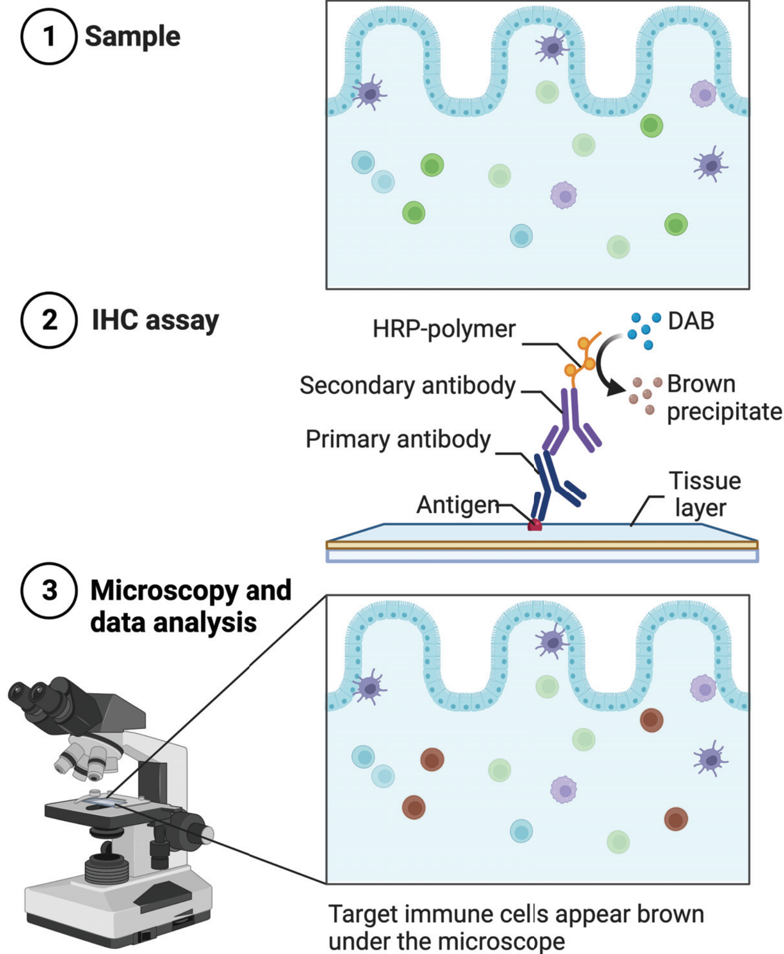

Figure 8. The basic principle of immunohistochemistry
A primary antibody is applied to a tissue section, followed by incubation time to allow the antibodies to recognise and bind to their targets. Unbound and extra antibodies are rinsed away after this step, and the secondary antibody is applied. It contains a linker molecule containing horseradish peroxidase (HRP) enzyme. Following that, a chromogen or dye such as 3,3' Diaminobenzidine (DAB) is added. The HRP enzyme converts the DAB substrate into a brown precipitate that is formed in the tissue at the reaction site, providing a visual picture of where the primary antibody originally bound to its target. *Figure created by Biorender.com*

Multiplex Immunohistochemistry (mIHC)

The ambition to see several antigens in a single tissue segment is almost as old as IHC itself. Nakane and Pierce (1967)[380] addressed the possibility of multiple stainings when they described their first immunoenzyme single-staining approach. Indeed, one year later, Nakane (1968) presented a multiple-staining method that used three indirect immunoperoxidase procedures successively differentiating the localization of three different antigens by three colors in a single tissue segment.[381]

With advances in the field of immunotherapy against cancer, there has never been a greater need for understanding the immunological or tumor microenvironment. Cells do not occur or act in isolation, and cellular responses often involve activation of several pathways that impact the type of their responses. Visual readings of IHC stainings suffer from high interobserver variability, which may be lessened by the application of computerized signal quantifications. Thus, the field is transitioning away from single-marker IHC towards multiple marker detection using digital image analysis.

Multiplex immunohistochemistry (mIHC), also known as multiple immunolabeling or multiplex immunostaining, increases the amount of information derived from a single tissue section. It allows for visualization of multiple targets on the same tissue section, as well as making analyses of spatial relationships possible. The mIHC technique includes the application of several labeled primary antibodies, spectral separation of fluorochromes, bleaching of fluorophores or chromogens, and blocking of previous antibody layers, in various combinations (figure 9). Multiple staining on the same section can be performed using species-specific secondary antibodies connected with a reporter, directed against antibodies generated in different animals (rabbit, goat/sheep, rat, and three to four mouse isotypes). Due to the limited availability of antibody sources, multiplexing techniques to stain antibodies generated in the same species with differing colors are inevitable. This can be managed in three ways: 1) bleaching directly conjugated primary antibodies before adding other layers; 2) preventing access to a previously deposited antibody for a second staining cycle; or 3) removing antibodies from sections after staining and imaging.[382]
The main barriers to widespread use of this technology are the high cost of unique antibodies and of required equipment, the limited throughput, and the shortage of trained people and specialized facilities.

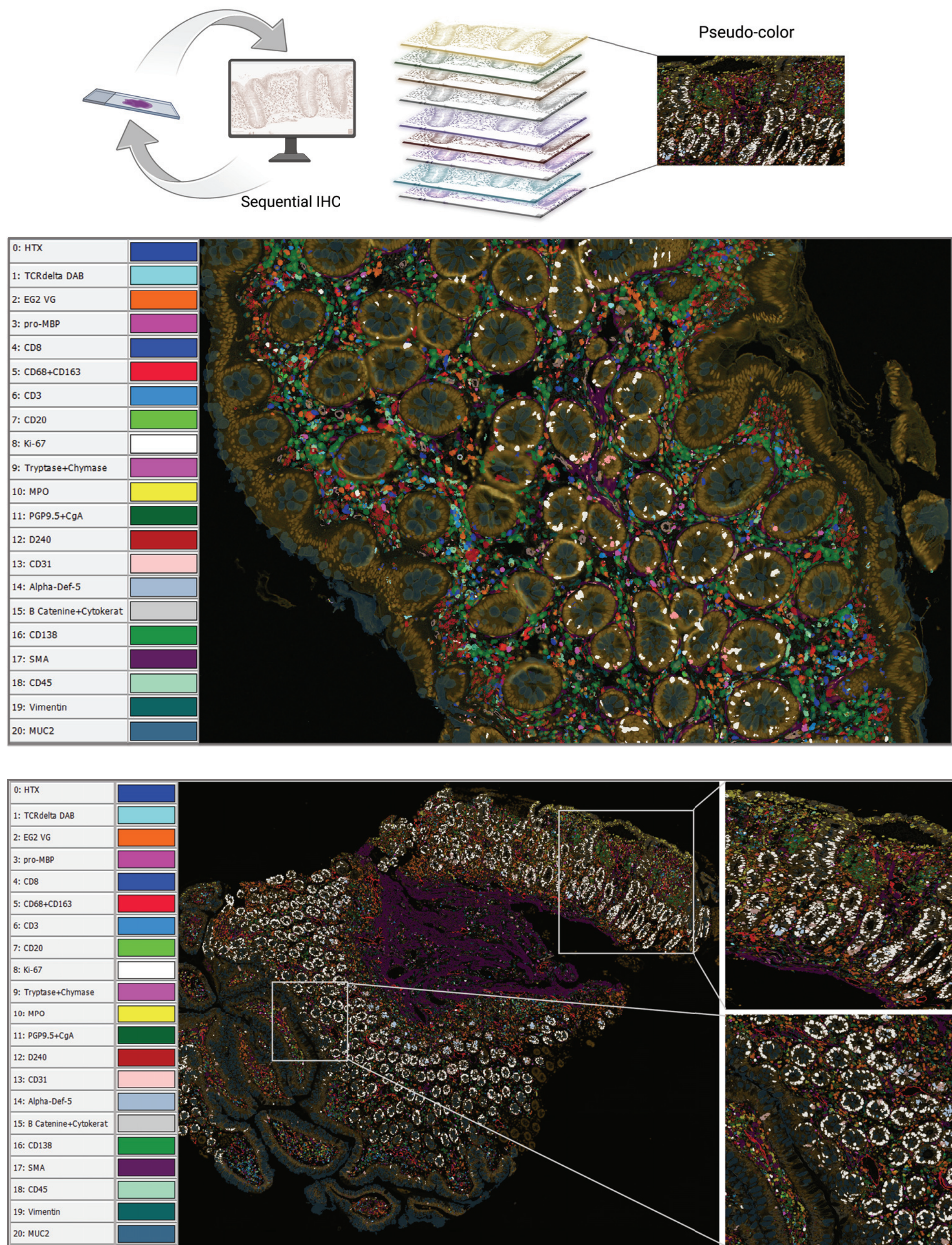

Figure 9. The principle of multiplex immunohistochemistry (mIHC) with examples of mIHC processed IBD tissue samples. Top image describes the basic workflow of mIHC. Middle and bottom panels show images of FFPE intestinal sections from UC and CD patients, respectively, where multiple stained targets are seen on the same tissue sections. On the left is the key for the colorcoded targets.

3. Quantitative image analysis

Quantitative image analysis (QIA) refers to a specific field that is designed to extract meaningful information from images in an objective and consistent manner. It allows for histological section analysis that is not feasible by visual measurements and mitigates the problem of inter- as well as intra-observer variability. Several procedural steps, each of which is optimized, are necessary for optimal readout, including image acquisition, image processing, image analysis, and image data visualization (Table 1).

Table 1. Images and data

	Input	Output	Examples
Image processing	Image	Image	Background subtraction; Contrast enhancement; Tissue segmentation
Image analysis	Image	Numbers	Cell count; Intensity
Image data visualization	Numbers or image	Image	Plots and graphs

Imaging in immunohistopathology

After staining the tissue section detecting target biomolecules and cellular components, the staining reaction produces visual signals that are viewed as images. There are two main types of image viewing devices: microscopes and whole slide scanners. Viewing the stained slides under a light or fluorescence microscope with the naked eye will generate qualitative data about the target. While this method provides important details regarding the samples, it is time consuming and relatively imprecise.

Whole slide scanning has transformed the field of digital histopathology. The digital image consists of a limited sized matrix of structure elements, usually referred to as *pixels* coming from picture elements. Digitization of the whole sample has many benefits including making viewing possible with more powerful tools than the microscope. Another advantage is transferability and storage. Images can be remotely accessed, both in clinical routine practice and research settings, facilitating collaborations.

Image processing and analysis

Before starting any image analysis workflow, one must establish that the tissue, histology slide, stain, and scan are of sufficient quality that it allows for the acquisition of meaningful and reproducible data. The validity of image analysis data might be substantially impeded by analysing low-quality tissue or sections, or stainings that are not well optimized. Factors such as the time between tissue harvest and fixation, as well as the length and intensity of fixation, are frequently poorly controlled.

Image processing and analysis relies on specialized software for computers which assists in the identification of regions of interest by either intensity, color, size, or form, enabling the user to extract relevant data from stained tissues.

Applying regions of interest (ROI) is regularly done to keep image analysis regions to smaller areas. Whole tissue images include large quantities of information that can be challenging to process and interpret as a whole. Furthermore, not every area of the entire slide image is necessarily informative. Expansions of parts of tissue, for example, may have the effect of diluting relevant information. Depending on the aim of the analysis it is advantageous or not to analyze the whole tissue. In most of our investigations we analysed whole tissue as it was relevant for achieving the most accurate quantification of various immune cells in a given condition. The software analyzes all of the tissue included in the inclusion annotation, but disregards from tissue regions designated with exclusion ROI annotations. Exclusions may be motivated by tissue features that are not suitable for examination, tissue artefacts, and staining artefacts.[383]

The computer image analysis software most often guides the user. Depending on the level of automation and the available algorithms, the software can perform various computations regarding cells or cellular components, e.g. the total quantity of stained cells in a specific region, or the proportion of stained area versus unstained area in a sample. It is possible to have the software segmenting out the ROI based on contrast, color, or color intensity. This function has been used in our work allowing us to segment out the LP from the epithelium digitally in intestinal tissue section images and analyzing each compartment separately (Figure 10).

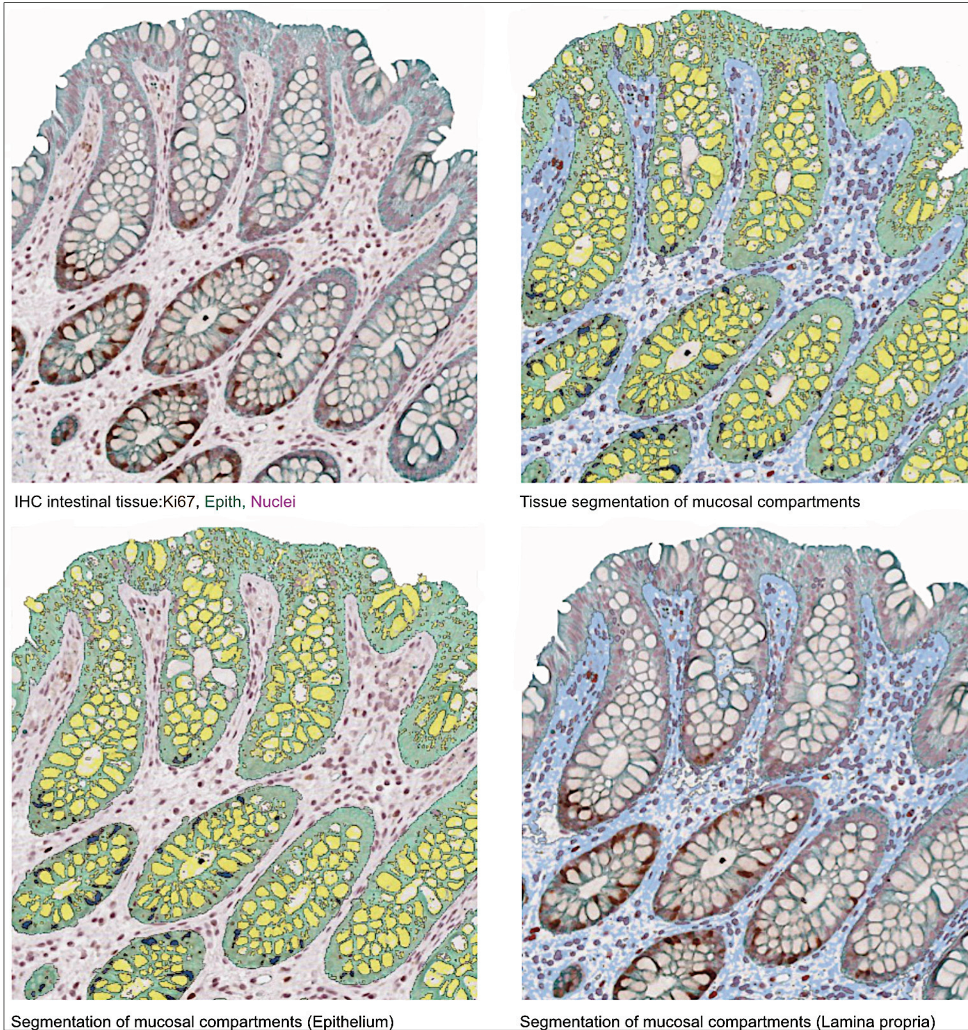

Figure 10. Quantifying Ki67 in the whole tissue, epithelial layer (EL) and lamina propria (LP)
The figure displays the process of automated tissue segmentation of an intestinal mucosal sample based on color recognition. The aim of this specific analysis was to quantify Ki67$^+$ cells in the whole sample, the epitheluim, and the LP, respectively. The first image (top left) shows the IHC staining before image processing and analysis, showing the digitized scanned slide stained for Ki67 (DAB, brown) and the epithelium (Vinagreen, green) identified by antibodies to cytokeratin and β-catenin, with the nuceli stained purple by hematoxylin for contrast**.** Tissue segmentation of mucosal compartments (top right) demonstrates the different parts of the tissue identified by the software with quantification of the Ki67$^+$ cells in the whole tissue section. The software applies new colors such as green for epithelial cells, yellow for goblet cell spaces, and blue for the LP. (Lower right) demonstrates the seperation of the LP from the epithelium by instructing the software to omit the epithelial green signal and quantify only the LP Ki67$^+$ expression (in red/violet). (Lower left) demonstrates the quantification of Ki67$^+$ expression in the epithelial compartment only (in dark blue).

Summary of papers

Paper I: Alkaline sphingomyelinase (NPP7) impacts the homeostasis of intestinal T lymphocyte populations.

Nucleotide pyrophosphatase/phosphodiesterase 7 (NPP7), also known as alkaline sphingomyelinase (Alk-SMase), is an enzyme expressed mainly at the small intestinal brush border.[384] NPP7 mediates hydrolysis of sphingomyelin (SM), a type of sphingolipid that is a constitute of mammalian cell membranes and is abundant in dietary components such as cheese, egg, meat, and milk.[385] Knockout (KO) mice have been generated to study the effects of NPP7 activity. It is known that ceramide and sphingosine-1-phosphate (S1P) are important bioactive metabolites that are downstream of NPP7 activity. These metabolites have been previously shown to have roles in cell trafficking, signal transduction, and apoptosis.[386,387] When NPP7 KO mice have been used in animal models for IBD and colorectal cancer, the KO mice displayed more prominent disease.[322,388] However, a basic immunological characterization of the NPP7 KO mice under homeostatic conditions has not been performed. Since the enzyme is primarily expressed in the gut, it would be expected that potential homeostatic immunological changes due to NPP7 deficiency would be best studied in the gut and related lymphoid tissues. We therefore applied quantitative IHC image analysis to investigate potential effects on the immune system of the small and large intestines, the mesenteric lymph nodes, and the spleen in NPP7 heterozygote and homozygote KO mice, respectively, and WT littermates. Immune cells studied were $CD3^+$ T-cells, $CD8^+$ cytotoxic T-cells, $CD4^+$ helper T-cells, $Foxp3^+$ T_{regs}, $B220^+CD19^+$ B-cells, $CD138^+$ plasma cells, mucosal IgA producing cells, $F4/80^+CD163^+$ macrophages, $F4/80^-CD163^-CD11c^+$ DCs, $CD3^-$ $Zap70^+$ natural killer (NK) cells and MPO^+ neutrophils.

Main findings

- NPP7 KO mice have increased numbers of T-lymphocytes in the small intestinal mucosa, whereas DC numbers are decreased.
- Lack of NPP7 has similar effects on T-cell populations in the large intestine as in the small intestine.
- Mesenteric lymph nodes of NPP7 KO mice display a reduced numbers of T-lymphocytes and dendritic cells.
- NPP7 deficiency does not affect lymphoid or myeloid cell populations in the spleen.

Discussion

In the current study we applied quantitative IHC image analysis to quantify major immune cell populations in the small and large intestines, MLNs, and spleens of NPP7 heterozygote and homozygote KO mice, respectively, as well as of WT mice, with the intent of investigate a potential role for NPP7 in the homeostasis of the gut immune system, which plays a part in several diseases such as inflammatory bowel disease and colorectal cancer.

The numbers of the T-cells were increased in the small intestinal mucosa and colonic mucosa in the KO mice compared to WT mice. In contrast, the numbers of T-cells were similar or decreased in the MLN of NPP7 KO mice. The number of DCs in NPP7 KO mice were significantly decreased in the small intestine but did not change significantly in the MLNs, although the was a numerical trend towards decreased numbers. Other immune cell populations were similar between the three genotypes. The results are strengthened by the fact that the number of animals examined is relatively high for this type of study. In addition, the fact that both heterozygote and homozygote animals were examined, and that several cellular analyses were suggestive of a genetic dose-response relationship further reinforced the results. Collectively our data demonstrate an important role for NPP7 in the regulation of T-cell homoeostasis in the intestinal mucosa.

Previous studies showed that NPP7 was reduced in patients with colonic adenocarcinoma.[389] Furthermore, patients with UC had decreased colonic NPP7 levels compared to controls,[390,391] and in a rat DSS-colitis model, rectal administration of recombinant NPP7 was shown to relieve colitis.[392]

Several mechanisms could potentially link our findings to known functions of NPP7. PAF, which is a mediator of inflammation, has been shown to be inactivated by the NPP7.[393] Furthermore, mucosal lysophosphatidic acid (LPA) and autotaxin have been shown to be increased in NPP7 KO

mice.[394,395347]Absorbed sphingosine is transformed to S1P in the mucosa by the sequential action of NPP7 and mucosal ceramidase. Some of the S1P produced may have effects in the mucosal compartment via paracrine receptor-mediated and direct intracellular signaling, regulating lymphocyte recruitment. If NPP7 activity indeed leads to increased S1P levels in the mucosa, it is simplistically paradoxical that knocking out NPP7 would lead to an increased influx of T-cells. However, the modulation of S1P levels in tissues is complex, involving numerous distinct metabolic pathways that can be altered at either the production or breakdown stages. In addition, as mentioned earlier, there are also data to suggest that S1P has a protective role locally in the gut mucosa, supporting gut barrier function and homeostasis.[333,337,338,396]Changes in T-cell numbers in the intestinal mucosa and draining mesenteric lymph nodes in NPP7 KO mice could potentially be directly or indirectly connected to variations in S1P levels.[385,397] When taken together, NPP7 KO mice show a predisposition to development of inflammation. Further research will be required to dissect the exact sequences of events that link NPP7 activity to intestinal T-cell numbers and inflammatory activity, including time course studies on immune cell populations, measurements of gene and protein expression, and examinations of levels of lipid messengers and various enzymes involved in sphingosine metabolism in the various anatomical and tissue compartments, in homeostatic and inflammatory conditions.

Paper II: Inflammatory bowel disease activity measured by quantitative immunohistochemical image analysis of innate immune cells

Histological examination of mucosal tissue in IBD is a sensitive tool to measure disease activity, and histological remission is emerging as a potentially important treatment target.[279]There are several existing histopathological indices, but they often encompass caveats such as not primarily having been designed to measure the degree of inflammation, including subjective components with poor intra- and interindividual reproducibility, and requiring expert pathologists that are scarce, resulting in extended response times. The aim of this study was to construct a new computerized and automated index for measuring histological disease activity non-

subjectively in both ileal and colonic mucosa, performing well for both CD and UC.

Main findings

- Computerized IHC image analysis quantification of epithelial neutrophils and macrophages, and LP neutrophils, in intestinal tissue samples separates IBD patients from control subjects.
- We were able to construct a new computerized and automated index (QiC3) for measuring histological disease activity non-subjectively functioning for both ileal and colonic mucosa, and for both CD and UC, based on the number of epithelial neutrophils and macrophages, and LP neutrophils.
- The QiC3 index has the ability to differentiate IBD patients with active disease from control subjects and from IBD patients with inactive disease.
- The QiC3 correlates significantly with tissue expression levels of proinflammatory mediators.

Discussion

Histological evaluation of mucosal biopsies is a sensitive tool to evaluate IBD activity. Mucosal healing is considered an important treatment goal in IBD because it is associated with better long-term outcomes and improved disease control.[398–400] Mucosal healing entails absence of inflammation and restoration of the normal architecture of the intestinal lining.

Ileocolonic biopsies were collected from healthy controls, and patients with CD or UC. A group of CD patients were sampled before and after 12 weeks of anti-TNFα therapy. Another group of CD and UC patients served as a small validation cohort. We examined three subsets of immune cells (i.e. neutrophils, macrophages, and T-cells) in two microanatomical mucosal compartments, i.e. the epithelium and the LP, to construct a new immunohistological index. We selected these subsets of immune cells since it is well-known that neutrophils infiltrate into the intestinal mucosa of IBD patients,[401] since the frequency of proinflammatory macrophages has been shown to be elevated in the inflamed mucosa of IBD patients,[402] and since there are strong indications that mucosa-infiltrating proinflammatory T cells drive disease activity in IBD.[403] Our results showed a significant increase in the numbers of epithelial and LP neutrophils, as well as epithelium-associated

macrophages, but not T-cells in mucosal samples from IBD patients compared to healthy controls.[245,404] This latter finding was somewhat surprising, however, there are indeed other recent reports that also have observed an absence of increased mucosal T-cell numbers in IBD, alternatively not consistent elevations in T-cell numbers, and for some T-cell subsets even a decrease.[245,404,405] In line with our data, there are also other recent studies that have highlighted neutrophils as the most important cell-type for separating between inactive and active IBD even though other immune populations are also involved.[406–408]

The immune cell types within the epithelium and the lamina propria, separately, were enumerated, and the numbers were compared between healthy controls and patients with CD or UC, respectively. The numbers of neutrophils and macrophages in the epithelium, and neutrophils in the lamina propria exhibited the best sensitivity and specificity for separating healthy control tissues from CD and UC tissues. These three parameters were thus chosen to construct a new index, named QiC3 1.0, that could separate tissues from healthy controls and patients with CD or UC with high precision. It performed equally well in a small validation cohort of patients. The QiC3 index correlated well to previously described histopathological indices GHAS (CD) and GS (UC) and showed worse correlation with endoscopic and symptomatic scores. Applying the new index to tissues from CD patients before and after therapy, it showed good responsiveness as it described a distinct amelioration in the microscopic inflammatory status, which also corresponded well to histopathological score improvements. In conclusion, we here describe a new quantitative, computerized, automated, non-subjective, and response-sensitive immunohistological index for measuring disease activity in both ileal and colonic mucosal biopsies, which is suitable for both CD and UC.

Paper III: Dissecting the heterogeneity of inflammatory bowel disease applying quantitative multiplex immunohistochemical image analysis

Heterogeneity among IBD patients is of a magnitude that seriously hampers optimal and personalized treatment as well as the developmental work striving for an improved understanding of the pathogenesis and development of efficient treatment strategies. There is a clear unmet need in the field of IBD with regards to therapeutic options and efficacy, together with a lack of biomarkers that predict therapeutic response and disease prognosis. Within each subcategory of IBD, i.e., CD, UC, and IBD-U, there is strong heterogeneity which could be described as a continuum, or alternatively as a number of disease subtypes. In IBD diagnostics

and clinical practice there are no feasible molecular or immunological correlates to differentiate such disease subtypes. A key explanatory element for this is most likely an immense heterogeneity in disease-related genetics, gut microbiome, and environmental factors that the patients have been exposed to. Little is known about how immune cells interact with one another and the epithelium in the mucosa of IBD patients. Thus, an immune-based histopathological classification with high resolution is lacking.

The aim of this study was to examine the cellular composition, including immune cell populations, of the intestinal mucosa in patients with IBD. We performed a multiplex immunohistochemical image analysis-based quantification of epithelial, stromal, and immune cell populations with analysis of spatial relationships in intestinal mucosal biopsies from patients with CD or UC, and from control subjects.

Main findings

- The number of neuroendocrine cells was reduced in both ileal and colonic epithelia in CD patients.
- The number of colonic subepithelial α-SMA$^+$ cells in both CD and UC patients.
- Cells in the LP in IBD display increased proliferative activity as assessed by Ki67 staining.
- The lymphatic vascular system is in disarray in IBD.
- In ileal biopsies from CD patients, there is a significant neural decrement.
- In inflamed mucosa from both CD and UC patients, there are decreased numbers of γδ T-cells and CD8$^+$ T-cells.
- Mucosal neutrophil numbers were increased in both CD and UC patients with active disease.
- Eosinophil numbers tended to be most increased in biopsies with non-active disease.
- Mucosal IBD biopsies showed a high degree of variability regarding cell clustering tendency, as well as considerable differences between cell types in terms of colocalization tendency.

Discussion

IBD is characterized by heterogeneity in the location of inflammation, disease behaviour, clinical course, and response to treatment. This poses challenges for

clinicians in diagnosing and managing the condition effectively. It highlights the importance of individualized treatment plans and continuous monitoring to address the specific needs and characteristics of each patient with IBD.[409] More than 240 genetic risk loci for IBD, as well as external risk factors, including lifestyle, stress, smoking, food, dysbiosis, and some drugs including NSAIDs, have been identified. Once the diagnosis is established, additional factors influencing heterogeneity are introduced, such as disease duration, past and current IBD medications, surgical resections, and other concomitant diseases. When attempting to address the heterogeneity of IBD, these circumstances are humbling, but they also highlight the importance of attempting to dissect it and they motivate the application of novel advanced methodologies designed to gain high-resolution pathophysiological information and/or to combine several omics data modules.[410]

Epithelial proliferation was not significantly higher in CD and UC compared to healthy epithelium. However, there was a numerical trend indicating greater epithelial proliferation, which has been described by others.[404] However, LP Ki67 expression was increased in inflamed colonic LP and correlated with the severity of inflammation. This observation is supported by research showing that activated T- and B-cells contribute to mucosal cellular proliferation in IBD and in murine colitis models.[120,411]

The expression of MUC2, which is one of the principal mucins produced by goblet cells, did not differ significantly between IBD and healthy tissue in our study, although there was a numerical trend towards decreased expression in IBD. A limitation in this context is that we only looked at one type of mucin, while there are several others that also have been linked to gut inflammation, namely MUC1, MUC3, and MUC4.[149] It is also possible that impacts on goblet cells and mucin production are important at different stages of a flare or at different levels of inflammation.[35] α-Defensin-5 expression in inflamed tissue did not differ from that in healthy tissue in our study. However, evidence indicating lower α-Defensin-5 levels have previously been reported. It is unclear whether these changes are primary, caused by genetic flaws in NOD2 that result in impairments in defensin synthesis, or secondary, caused by the loss of intestinal epithelial cells, including Paneth cells, during active inflammation.[412]

The inflammatory milieu in CD varies substantially between the colon and the ileum. We discovered an unusually diverse pattern in our original raw histomic data. Surprisingly, the epithelial compartment exhibited the majority of the substantial differences when compared to healthy tissues. For example, a decrease

in intraepithelial neuroendocrine cell numbers were observed in both ileal and colonic CD but not in UC. Serum chromogranin A, interestingly, has previously been suggested to function as a biomarker in CD.[413] A possibly related result in our study was a decrease in the presence of neuronal cells in the LP in CD.[414]

Fibroblasts are known to play an essential role in IBD inflammation.[415,416] The number of LP vimentin^{+} fibroblasts declined across the IBD sample groups and corresponded inversely with disease severity. Subepithelially located α-SMA^{+} myofibroblasts were similarly reduced. Fibroblasts have been hypothesized to be replenished after injury but the discovery of a decrease in vimentin expression in IBD is novel, and more research into this topic and the role of these cells in IBD is needed to better understand its significance.[417] Finally, we found that podoplanin (D2-40) expression changed in both quantity and quality. In certain biopsies, the normally well-organized network of lymphatic vessels was shown to be completely disorganized. This could be very essential in the pathophysiology of IBD and warrants more research.

In our study we found that the number of CD4^{+} IELs increased in colonic CD and UC, and this increase correlated with disease activity. CD8α^{+} T cells on the other hand showed a reduction in ileal CD and UC. Interestingly, murine studies have revealed a decline in CD8^{+} IEL counts in the early stages of illness.[418,419] In addition, other groups have recently described lower CD8^{+} T-cell numbers in the mucosa of CD patients compared with controls.[404] γδ IELs are believed to be microbiota-stimulated, to have a protective function in case of intestinal damage, to restrict inflammation in mouse studies,[244] and to release immunosuppressive cytokines such as TGF- and IL-10.[420] In our study, γδ IELs showed a definite decline in numbers in UC, which was inversely linked with the histopathological scores.

Our findings demonstrated a significant increase in epithelium-associated and LP MPO^{+} neutrophils across all IBD groups. Epithelium-associated mast cells were more prevalent, and their numbers corresponded with disease activity in ileal CD. Mast cells have also been shown to interact with gut microbiota, triggering them to become activated and degranulate, and to accumulate in inflammatory lesions.[421] Our density plot results indicate that subepithelial cell cluster presence varies, offering intriguing prospect to help explain the variability in mucosal subtypes of IBD. Additionally, a combination of a more thorough spatial study will be interesting to perform. In conclusion, our approach permits the simultaneous study of a large number of IBD-related cells and mediators in

combination with the application of several analytical techniques, each of which sheds light on the heterogeneity of IBD from various perspectives. The data primarily highlight the immense variability of IBD and simultaneously demonstrates that the existing methodology holds true potential, although our investigation has already at this stage revealed some novel and in and of themselves highly fascinating findings. Future studies will examine a number of additional cell type subgroups and additional inflammatory mediators after this first relatively coarse mapping, which along with more bioinformatic analyses will move us closer to understanding the heterogeneity of IBD.

Paper III: Diffuse alveolar damage patterns reflect the immunological and molecular heterogeneity in fatal COVID-19

Severe COVID-19 lung disease exhibits a high degree of spatial and temporal heterogeneity, with different histological features coexisting within a single individual. It is important to capture the disease complexity to support patient management and treatment strategies. We provide spatially decoded analyses on the immunopathology of diffuse alveolar damage (DAD) patterns and factors that modulate immune and structural changes in fatal COVID-19. Autopsy lung tissues of 18 COVID-19 patients were analyzed by means of multiplex IHC image analysis. Cytokine profiling, viral, bacteria, and fungi detection, and transcriptome analyses were also performed.

Main findings

- Spatial DAD progression was associated with expansion of fibroblasts, macrophages, $CD8^+$ T-cells, and lymphangiogenesis.
- Viral load was positively associated with exudative DAD and negatively associated with disease length and hospital stay.
- Enteric bacteria were isolated from lung tissue in all 18 cases, and *Candida parapsilosis* was isolated in 8 cases.
- Cytokine levels were positively associated primarily with macrophages and $CD8^+$ T-cells.
- Coagulative pathways and molecular repair systems were enriched in exudative DAD whereas intermediate or advanced DAD displayed a molecular profile of enhanced humoral and innate

immune response activity and elevated extracellular matrix production.

Discussion

The lungs can experience heterogeneous, viral-induced DAD in severe COVID-19 cases. Secondary events including thrombosis, pulmonary and cardiac infarctions, and non-viral infections together with life-supporting care add complexity to the immunopathological picture. To enhance disease management and maximize cutting-edge therapeutic approaches, it is crucial to better comprehend this complexity.

In this study we demonstrated that typical features of COVID-19 lung disease were strong proliferating responses, a patchy pattern of zones with exudative DAD, abundant in macrophages and neutrophils, and a high virus load, with numerous deferentially expressed genes (DEGs) connected to thrombotic events and acute cellular responses. These coexisted with various intermediate or advanced DAD regions, enhanced macrophage, lymphocyte, and DC responses, epithelial hyperplasia, lymphangiogenesis, structural transformation, a locally lower viral load, and DEGs associated with the production of Ig and extracellular matrix. Our findings of a connection between SARS-CoV-2 viral load and exudative, but not intermediate or advanced DAD areas, lends support to the proposed hit-and-run process, where the time duration of the virus tissue-damaging infection-replication-propagation sequence cycle is brief.[422] Patients with COVID-19-associated DAD had more proliferating cells than control subjects, as demonstrated by an increase in the total density of immune cells $CD8^+$ T-cells, macrophages, and mast cells. The density of structural cells such epithelial cells, endothelial cells, and fibroblasts increased as well. However, such total tissue quantification does not account for the variability and complexity of cell arrangements present in each lung biopsy and may explain the disparities in the literature, particularly in the quantification of lymphocytes.[423] In exudative DAD, epithelial and endothelial cell denudation predominates, whereas in the intermediate and advanced phases, distinct structural cell growth and growing cellular immune responses occur, along with a reduced viral load. Our findings reveal that the density of T- and B-cells increased in intermediate and advanced DAD and was associated with a peak of DC numbers in the intermediate phase that correlated negatively with viral load. Previous research has shown an increase

in activated $CD4^+$ and $CD8^+$ T-cells in the lungs of critically ill COVID-19 patients.[424] Lymphocytic responses in COVID-19 have been extensively studied, and results point to an association between poor, excessive, or otherwise inappropriate T-cell responses and severe disease. In addition, substantial heterogeneity was found in $CD8^+$ T-cell gene expression in critically ill patients.[424,425]

Macrophages have an important role in COVID-19 immunopathology. Single-cell analyses revealed an inflammatory macrophage phenotype in the lungs of COVID-19 patients. Our findings reveal that *in situ* proliferation considerably contributes to the persistent increasing amounts of these cells in the lungs of critically ill patients, occasionally creating pseudopalisades of cells. Macrophage numbers correlated with various antiviral cytokines, such as interferons, lending credence to their participation in antiviral defenses. Furthermore, during COVID-19 ARDS, Wendisch *et al.* discovered a profibrotic transcriptional pattern in macrophages.[426] Little is known about the dynamics of DCs in human DAD, but the observed peak correlated with the expansion of B- and T-cells, hyperplastic alveolar epithelium, and local lymphangiogenesis. Taken together, these findings imply that DAD development is related with the establishment of a unique axis of reprogrammed alveolar epithelium, DCs, B- and T-cells, and lymphatics, a process that may offer a basis for enhanced adaptive immune responses with an increased spread to other organs. Endothelial cell abnormalities, angiogenesis with proliferating $CD31^+$ cells, and aberrant lymphangiogenesis were all associated with frequent thrombotic events. The increase in lymphatic vessels in the intermediate DAD may lead to the distribution of immune responses systemically, but on the other hand also to eliminate excess interstitial fluid which may constitute a clinically serious hazard in severe COVID-19. In conclusion, we have presented a comprehensive approach to the spatially and temporally heterogeneous immunopathology of lung involvement in severe cases of COVID-19. We have demonstrated that complex immunological microenvironments with different lung responses can coexist in the same patient, associated with the presence of secondary infections, thrombotic phenomena, and a cytokine rich milieu driven by different gene expressions. This was done by dissecting the different immune and lung structural arrangements within the three pathologically identifiable DAD patterns. Our findings supplement and deepen the understanding of other important studies such as single-cell RNA sequence analysis investigations that however lack the spatial context of the diseased tissue landscape. The vast

complexity illustrated by our study should be taken into account when offering new explanations to COVID-19-related pathophysiological processes and when suggesting or testing novel treatments with very specific targets.

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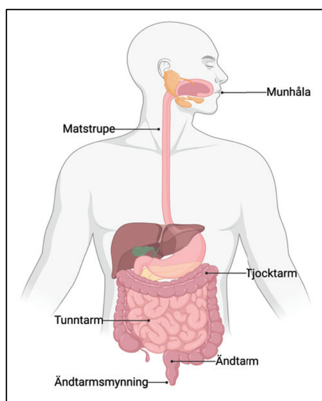
والحَمْدُ لِلَّهِ رَبِّ العالَمِينَ حَمْداً يُوافِي نِعَمَهُ ويكافئ مَزيدَهُ.

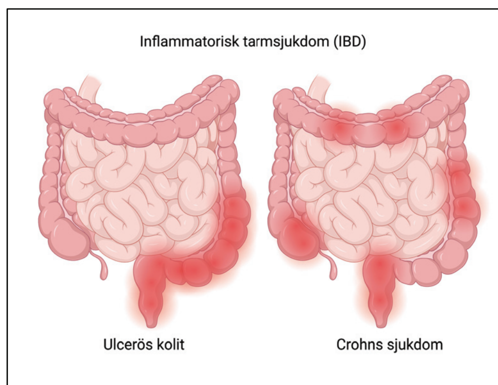
Populärvetenskaplig sammanfattning

Immunsystemet skyddar tarmarna (figur 1) och kroppen från att bakterier och virus sprids från avföringen till tarmväggen eller vidare till resten av kroppen. Immunsystemet bekämpar även cancerceller som kan uppstå i tarmens slemhinna. Samtidigt tillåter immunsystemet att goda och för kroppen fördelaktiga bakterier överlever inne i tarmen. De goda bakterierna bidrar också till att utveckla kroppens immunsystem, att underhålla och balansera det. Om denna balans sätts ur spel kan immunsystemet bli överaktivt och aggressivt, och börja angripa de goda bakterierna. I denna kamp skadas slemhinnan och man utvecklar så kallad inflammatorisk tarmsjukdom som förkortas IBD utifrån den engelska benämningen inflammatory bowel disease. Det finns två huvudtyper av IBD, Crohns sjukdom (Crohn's disease, CD) och ulcerös kolit (ulcerative colitis, UC). De exakta orsakerna till varför vissa utvecklar IBD och vilka steg på vägen som sker är inte helt klarlagt, men man vet att det krävs en viss typ av genuppsättning som gör vissa mottagliga för att utveckla IBD, i kombination med inte helt klarlagda miljöfaktorer, samt en viss typ av sammansättning av bakterieflora i tarmen. Det finns i dagsläget inget botemedel, men med hjälp av intensiv forskning har man tagit fram allt bättre läkemedel som kan stilla inflammationen i tarmen och därmed sjukdomsaktiviteten. Många personer med IBD blir dock inte bra av dagens möjligheter till behandling och behovet av fortsatt forskning är stort.



Figur 1. Matsmältningskanalen

Vid Crohns sjukdom kan vilken del som helst av mag-tarmkanalen drabbas, från munnen till ändtarmen, men det vanligast stället som påverkas är nedersta delen av tunntarmen. Ulcerös kolit drabbar nästan alltid ändtarmen och olika mycket av tjocktarmen men inte övriga delar av mag-tarmkanalen (figur 2). Vanliga symtomen vid IBD är ihållande diarré, blod i avföringen, buksmärta, viktnedgång och trötthet. Komplikationer till följd av sjukdomen kan uppstå i form av fistelbildningar (små millimeterstora gångar som utgår från den inflammerade tarmen och penetrerar omkringliggande vävnader), varbölder i buken eller runt ändtarmen, strikturer (förträngningar av tarmen som kan hindra att avföring kommer igenom), och tjocktarmscancer. IBD behandlas med olika läkemedel som har det gemensamt att de dämpar eller balanserar immunsystemet. Ibland krävs det att man kirurgiskt avlägsnar den inflammerade delen av tarmen. Ibland, men alltmer sällan, behöver man anlägga en stomi (påse på magen).

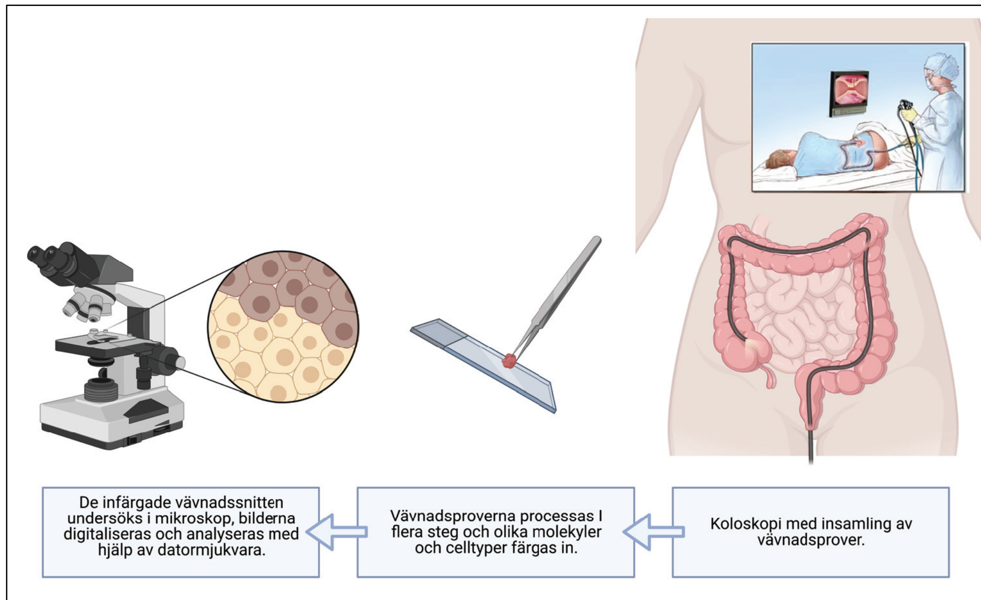


Figur 2. Inflammatorisk tarmsjukdom (IBD)

Diagnosen ställs genom en sammanvägning av flera faktorer, så som symtom, avföringsprover, blodprover, koloskopi (undersökning av tarmen med en slangkamera), undersökning av vävnadsprover från tarmslemhinnan, och ibland någon typ av röntgenundersökning (skiktröntgen, magnetkamera eller ultraljud). Man ska också ha uteslutit andra orsaker som till exempel en infektion (bakterier, virus och tarmparasiter).

Vävnadsproverna som samlas in i samband med en koloskopi kan, förutom att användas i kliniskt syfte där en klinisk patolog undersöker proverna, användas för forskning, vilket är det vi har gjort i denna avhandling (figur 3). Med så kallad immunhistokemi kan man färga in vävnadsproverna för olika molekyler och celltyper. Med nya tekniska hjälpmedel kan man digitalisera bilderna från vävnadssnitten och analysera dem med hjälp av datormjukvara och artificiell intelligens. De olika molekylerna och celltyperna kan på så vis kvantifieras mer exakt och man kan analysera de spatiala förhållandena mellan olika celltyper, det

vill säga att man kartlägger var de olika cellerna, molekylerna och vävnadsstrukturerna befinner sig i förhållande till varandra i det mikroskopiska vävnadslandskapet. Detta ger information om olika typer av sannolika interaktioner och mekanismer för hur immunsystemet fungerar och varför det uppstår sjukdomsalstrande inflammation.



Figur 3. Koloskopi med insamling av vävnadsprover.
Figure created by BioRender.com

I det första projektet använde vi denna teknik för att undersöka hur tarmens immunsystem såg ut hos möss som fått ett enzym i tarmens borstbräm, NPP7, utslaget. NPP7 spjälkar i vanliga fall sfingomyelin som är en vanlig fettyp i vissa födoämnen (till exempel mjölk, kött och ägg). Det visade sig att mössen med utslaget NPP7 hade en klar ökning av en viss typ av vita blodkroppar som kallas T-celler i tarmslemhinnan. Således verkar NPP7 till att bidra med att dämpa och balansera tarmens immunsystem.

I det andra projektet använde vi samma teknik för att ta fram en mått på hur kraftig inflammation som råder i tarmen slemhinna, genom att kvantifiera vissa subtyper av vita blodkroppar som kallas neutrofiler och makrofager i vävnadsproverna. Det kan vara ett bekymmer att man inte vet exakt vilken grad av inflammation som patienten lider av och därför kan ett sådant mått vara användbart, både innan man ska sätta in behandling för att veta hur kraftfull behandling som är adekvat, samt

för att utvärdera efter en tids behandling i vilken utsträckning behandlingen varit effektiv och om man ska fortsätta med samma medicin, ändra dosen eller byta till en annan typ av medicin. Ett sådant mått kan också vara användbart i kliniska prövningar av nya mediciner där man vill ha så exakta och stringenta utvärderingsverktyg som möjligt.

I det tredje arbetet använde vi återigen samma teknik, men denna gång färgade vi in för ett stort antal markörer av olika slag, närmare bestämt 23 stycken. Tanken med detta projekt är att karakterisera det komplexa immunsvaret som man ser i tarmvävnadsproverna. Hypotesen är att det finns långt fler undergrupper av IBD än bara Crohns sjukdom och ulcerös kolit, och om vi skulle kunna karakterisera dessa olika undergrupper närmare skulle man bättre kunna skräddarsy handläggningen och behandlingen av patienterna, samt bättre förutspå om patienten svarar på en viss behandling eller inte och hur sjukdomen kan komma att utveckla sig över tid vilket skulle kunna väga hur kraftfull behandling patienten bör ha redan från början. Att behandla alla patienter med den kraftfullaste typen av behandling är inte önskvärt eftersom ju kraftigare behandling desto fler biverkningar och komplikationer kan det uppstå. Därför vill man reservera den kraftfullaste behandlingen till de patienter som förväntas utveckla en svår sjukdomsbild över tid. Förutom dessa kliniskt användbara mål med projektet, är tanken också att resultaten ska hjälpa oss förstå de olika sjukdomsmekanismerna bättre, vilket i förlängningen skulle kunna leda till utveckling av nya och bättre läkemedel.

I det sista arbetet använde vi samma teknik för att undersöka lungvävnadsprover från personer som avlidit i COVID-19. Resultaten beskriver den oerhört komplexa immunologiska situation som råder i lungan på dessa patienter, som kan vara viktiga att beakta när man ska utveckla behandlingsmetoder vid svår COVID-19.

Som det ofta är inom medicinsk forskning så genererar genomförda forskningsprojekt nya intressanta frågor och målsättningar. Så är det också i detta fallet och för alla fyra projekt finns det nya, mer fördjupande ansatser och analyser som planeras för att ta ytterligare steg i kunskapsutvecklingen.

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