Macrophages in Crohn´s Disease

Innate immune cellular and molecular mechanisms driving intestinal inflammation and fibrosis

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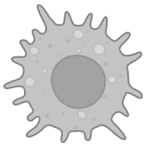

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ABSTRACT

Macrophages and their interactions with the lamina propria and luminal microenvironment are crucial in the pathogenesis of Crohn´s disease (CD), a chronic inflammatory disease with a strong inflammatory innate immune involvement. Therefore, interpreting macrophage activity in the intestinal microenvironment may identify treatment targets beneficial for at least a subgroup of patients. The overall aim of this thesis was to establish innate immune cellular and molecular mechanisms driving intestinal inflammation and fibrosis in CD. In more detail, it was aimed to determine the inflammasome components and TREM-1 receptor expression in CD in relationship to macrophage phenotypes, as well as to evaluate the effects of the CD fecal microenvironment on macrophages and fibroblasts phenotypes. The first paper showed that inflammasome component expression in CD was location- and cell-specific, and MEFV and NLRP3 inflammasome expression in ileal CD was attributed to the accumulation of immature macrophages. The second paper demonstrated that TREM-1 expression was higher in CD and attributed to increased numbers of immature macrophages increase in CD, defined as $CD14^{+}CD11c^{+}HLA\text{-}DR^{int/high}$, as well as lamina propria microenvironment changes in CD. The third paper established that the CD fecal microenvironment polarize the *in vitro* tissue-resident macrophages into a more pronounced anti-inflammatory phenotype while induce pro-inflammatory but no fibrosis-related changes on intestinal fibroblasts.

Overall, this thesis concludes that the increase of inflammasome and TREM-1 expression on macrophages, and the influence of fecal microenvironment on macrophages might be potential targets for treating CD. Forthcoming studies should aim to provide functional understanding and identify therapeutic targets in larger patient cohorts to meet the needs for improved treatments.

Keywords: macrophages, inflammatory macrophages, tissue-resident macrophages, efferocytosis, innate immunity, myeloid cells, fibroblasts, fibrosis, inflammasomes, Crohn´s disease, inflammatory bowel diseases, digestive diseases, intestinal immunity, mucosa, immunology, intestinal inflammation, serum systemic inflammation, microbiota, metabolites

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SAMMANFATTNING PÅ SVENSKA

Makrofagen är en celltyp som tillhör det medfödda immunförsvaret och spelar en viktig roll vid Crohn's sjukdom (CD), en kronisk inflammatorisk sjukdom i mag-tarmkanalen. Makrofager samspelar med tarmens mikromiljö och har en central roll i sjukdomens uppkomst. Genom att studera hur sjukdomen påverkas av makrofagerna i tarmen kan nya behandlingsstrategier som kan hjälpa vissa patienter utvecklas. Den övergripande målsättningen med denna avhandling var att fastställa cellulära och molekylära mekanismer inom det medfödda immunförsvaret som driver inflammation och fibros i tarmen vid CD. Mer specificerat var syftet att fastställa uttrycket av olika komponenter hos inflammasom-komplex och TREM-1-receptorn och deras relation till makrofagers egenskaper, samt att utvärdera effekterna av tarmens fekala mikromiljö på makrofager och fibroblaster vid CD. Den första artikeln visade att uttrycket av inflammasom-komponenter vid CD var plats- och cellspecifika, och uttrycket av MEFV och NLRP3-inflammasomen i tunntarms-CD kopplades till ackumulering av omogna makrofager. Den andra artikeln demonstrerade att uttrycket av TREM-1 var högre vid CD, vilket var associerat till ökat antal omogna $CD14^{+}CD11c^{+}HLA\text{-}DR^{int/high}$ makrofager, samt förändringar i mikromiljön i lamina propria. Den tredje artikeln fastställde att tarmens fekala mikromiljö vid CD polariserade alternativt aktiverade makrofager till en mer uttalad antiinflammatorisk fenotyp samtidigt som proinflammatoriska, men inte fibrosrelaterade, förändringar inducerades hos fibroblaster.

Den sammantagna slutsatsen av denna avhandling är att det ökade uttrycket av inflammasom-komponenter och TREM-1 hos makrofager samt den fekala mikromiljöns påverkan på makrofager kan vara potentiella mål för behandling av CD. Framtida studier bör syfta till ökad funktionell förståelse av sjukdomen samt identifiering av nya terapeutiska mål i större patientkohorter för att möta behoven av förbättrade behandlingsstrategier.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Gorreja F**, Caër C, Rush S.T.A, Forsskål S.K, Härtlova A, Magnusson M.K, Bexe-Lindskog E, Börjesson L.G, Block M, Wick M.J. MEFV and NLRP3 Inflammasome Expression Is Attributed to Immature Macrophages and Correlates with Serum Inflammatory Proteins in Crohn´s Disease Patients. Inflammation. 2022;45(4):1631-1650. doi:10.1007/s10753-022-01647-8.[1]

II. Caër C, **Gorreja F**, Forsskåhl S.K, Brynjolfsson S.F , Szeponik L, Magnusson M.K, Börjesson L.G, Block M, Bexe-Lindskog E, Wick M.J. TREM-1+ Macrophages Define a Pathogenic Cell Subset in the Intestine of Crohn's Disease Patients. Journal of Crohn´s and Colitis. 2021 Aug 2;15(8):1346-1361. doi:10.1093/ecco-jcc/jjab022.[2]

III. **Gorreja F**, Bendix M, Rush S.T.A, Maasfeh L, Savolainen O, Dige A, Agnholt J, Öhman L, Magnusson M.K. Crohn´s Disease derived fecal supernatants induce altered polarization of M2 macrophages and intestinal fibroblasts. 2023 (*Submitted manuscript*)

RELATED WORK NOT INCLUDED IN THIS THESIS

[3]The developmentally regulated fetal enterocyte gene, ZP4, mediates anti-inflammation by the symbiotic bacterial surface factor polysaccharide A on Bacteroides fragilis.
F. Gorreja, S.T.A Rush, D.L Kasper, D. Meng*, W. A. Walker*. American Journal of Physiology – Gastrointestinal and Liver Physiology. 2019 Sept 24; 317(4): G398–G407. doi: 10.1152/ajpgi.00046.2019.

[4]The potential role of adherence factors in probiotic function in the gastrointestinal tract of adults and pediatrics: a narrative review of experimental and human studies.
F. Gorreja, W. A. Walker. Gut Microbes. 2022 Dec 5; 14(1): e2149214. doi:10.1080/19490976.2022.2149214.

[5]Isobaric labeling-based quantitative proteomics of FACS purified immune cells and epithelial cells from the intestine of Crohn's disease patients reveals proteome changes of potential importance in disease pathogenesis.
J. Alfredsson, I. Fabrik, **F. Gorreja**, C. Caër, C. Sihlbom, M. Block, L.G. Börjesson, E. Bexe Lindskog, M. J. Wick. Proteomics. 2023 Dec 8 23(5) :2200366. doi:10.1002/pmic.202200366.

[6]Systematic review of human and animal evidence on the role of buckwheat consumption on gastrointestinal health.
E. Valido*, J. Stoyanov*, **F. Gorreja**, S. Stojic, Ch. Niehot, J. Kiefte-de Jong, E. Llanaj, T. Muka, M. Glisic. Nutrients. 2022 Dec 20; 15 (1): 1. doi:10.3390/nu15010001.

[7]Gene expression changes as predictors of the immune-modulatory effects of probiotics: Towards a better understanding of strain-disease specific interactions.
F. Gorreja. Nutrition and Food Science Journal. March 2019; 14–15: 1-5. doi:10.1016/j.nfs.2019.02.001

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Table of content

ABBREVIATIONS

GIT	Gastrointestinal tract
IBD	Inflammatory Bowel Disease
CD	Crohn´s Disease
UC	Ulcerative Colitis
iCD	inflammatory Crohn´s Disease
sCD	stenotic Crohn´s Disease
IECs	Intestinal Epithelial Cells
PRRs	Pattern-Recognition Receptors
LP	Lamina propria
MNPs	Mononuclear phagocytes
Mfs	Macrophages (tissue)
M1MQ	M1 Macrophages (*in vitro*)
M2MQ	M2 Macrophages (*in vitro*)
PBMCs	Peripheral blood mononuclear cells
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
NLRP	Nucleotide-binding oligomerization-Like Receptor PYD domain or Nucleotide oligomerization domain (NOD)-like receptor
MEFV	Mediterranean Fever
CASP	Caspase
CARD	Caspase activation and recruitment domain
IL (n)	Interleukin (number)
CCR (n)	Chemokine receptor 2
CCL (n)	Chemokine (C-C motif) ligand (number)

TLR	Toll-like receptor
TREM-1	Triggering Receptor Expressed on Myeloid cells 1
TNFα	Tumor Necrosis Factor alfa
TGFβ	Transforming Growth Factor beta
MCP-1	Monocyte Chemoattractant Protein 1
Pro-Col1A1	Pro-collagen I alpha 1
CD (n)	Cluster of Differentiation (number)
HLA-DR	Human Leukocyte Antigen – DR isotype
EpCAM	Epithelial Cell Adhesion Molecule
M-CSF	Macrophage-colony stimulating factor
α-SMA	alfa-smooth muscle actin
FAP	Fibroblast activation protein
ECM	Extracellular matrix
LPS	Lipopolysaccharide
FS	Fecal supernatants
MAMPs	Microbial associated molecular patterns
ELISA	Enzyme-Linked Immunosorbent Assay
RT-PCR	Real-Time Polymerase chain reaction
PEA	Proximity extension assay
MSD	Meso scale discovery
RNA	Ribonucleic acid
mRNA	messenger ribonucleic acid
DNA	Deoxyribonucleic acid
Ct	Cycle threshold
FMO	Fluorescence minus one
vs	*versus*

DEFINITIONS IN SHORT

Healthy human	The comprehensive definition of health according to the World Health Organization "Health is a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity"[8]. From a social perspective, personal definitions of health and being healthy are subjective. For the purpose of this thesis and research work I define healthy as merely the absence of a diagnosed and/or reported disease/condition/syndrome.
Homeostasis	Refers to a steady/stable state or normal functioning of a living organism.
Inflammation	A process during which the immune system intervenes, through inflammatory cells, to fight an infection or heal tissue injury/damage. An ongoing inflammation can be provisory (until the body is able to eliminate the infection/damage) or more long term (where there is absence of infections or injury but there is an ongoing chronic disease keeping inflammation process *on*).
Cytokine	Ample and diverse category of proteins characterize by a small size (ca. 5-25 kDa). They are important in cell-mediated signaling and immune responses. They interact with specific cytokine receptors on the same cell that produces the cytokine or nearby cells. Cytokines can also have effects on distant cells/organs (via the circulation), causing systemic effects of inflammation, for example causing fever or the production and release of acute phase proteins from the liver as well as release of neutrophils from the bone marrow.
Effector cells	In the immunology context effector refers to a cell which performs a specific action/function in response to a stimuli/trigger. An effector cell in other words is an active cell within a certain biological environment.

Mesentery	"*a fold of the peritoneum* which attaches the stomach, small intestine, pancreas, spleen, and other organs to the posterior wall of the abdomen*."[9] " **the serous membrane lining the cavity of the abdomen and covering the abdominal organs."*[9]
Short lived and long lived cells	In immunobiology characterizes the sustained presence of a cell. These expressions are used frequently for plasma cells but for some innate immune cells there is grey area in defining whether they are short or long lived. This is due to similarities between subsets that make it challenging to distinguish whether a cell is derived from an older cell or just polarized differently.
Phagocytosis	Phagocytosis is a function of many immune cells (typically macrophages and neutrophils but also dendritic cells), also called phagocytes, which engulf large particles (including microbes) by means of their plasma membrane with the aim to eliminate them to clear up the environment.
Efferocytosis	The term refers to the process of clearance of apoptotic (dying) cells in our bodies. It is a function performed by professional phagocytes (mainly macrophages and dendritic cells) and non-professional cells with low phagocytic capacity (epithelial cells, fibroblasts). This function is essential for the maintenance of tissue homeostasis (clearance).[10,11]
Complications	A medical complication is a predictable or unpredictable result of a disease, syndrome, or treatment of these. Complications affect the quality of life as well as the severity of the disease as they lead to new symptoms or even new diseases related to the initial one.
Receptor	In the immunobiology context and this thesis, a receptor is a molecular structure, typically of proteinic nature, that senses and converts signals presented to them. The ligands

	activating a receptor, and hence triggering a physiological response in the cell, can for example be bacterial components, small chemical molecules, proteins and/or cell metabolism byproducts.
Apoptosis	Apoptosis is a manner of controlled cell death. The process starts with blebbing (swelling), followed by a reduction in cell size, and nucleus component-related changes.
Pyroptosis	Pyroptosis is an inflammatory manner of controlled cell death happening by formation of plasma membrane pores and terminating in cell bursting.
++ and + labeling	*"The + denotes an expression level that is ~10-fold above the isotype control and ++ is ~100-fold above the isotype control."*[12]
"high", "int", "dim" labeling	Also used in flow cytometry to refer to expression levels of a specific cell marker on cells that are positive for that marker. "high" meaning a marker is highly expressed in those cells, "int" meaning an intermediately expressed, "dim" meaning a marker is lowly expressed. All compared to a "normal" expression that is anyway context specific.
Hematopoietic	Refers to cells or proteins of hematopoietic origin: various factors that are involved in the creation of blood cells.
Pleiotropic	It refers to a protein which performs different functions and is involved in numerous immune reactions. A pleiotropic protein can show pro- or/and anti-inflammatory actions depending on the context: the cells that produce it, stage of the inflammatory process, the signaling pathway. Used to describe the IL-6 cytokine in this thesis, however, it is not a unique term to IL-6, as it is used for many other proteins and genes.
Subset	A part of a larger group of a specific immune cell type.

1 INTRODUCTION

With this chapter my aim is to touch upon relevant topics that were the focus of the research work included in this thesis. The introduction is meant to summarize and narrate the basics of the topic, focusing on the disease/cells/receptors under investigation.

1.1 THE HUMAN INTESTINE IN HEALTH

The human gastrointestinal tract (GIT) is one of the largest organs of the human body whose main function is to **absorb and digest** nutrients necessary for the functioning of the human body. The intestine, or lower part of the gastrointestinal tract, is also a barrier that helps keep pathogens and our own microbiota separated from the underlying tissue. In general terms, the **intestinal mucosa** is formed by a single layer of intestinal epithelial cells (IECs), immune cells and stromal cells. IECs are covered by a mucus layer, above and within which the intestinal microbiota is found (Figure 1) as one of many communities of microbes found in human mucosas (e.g. intestinal, skin, airway). The barrier is formed in four main ways. First, the intestinal mucosa forms a physical **barrier** with the IECs covered by mucus which is more or less dense depending on the section of the intestine considered.[13] Second, the barrier can be biochemical meaning that bile and gastric acids besides helping in digestion can aid killing of microorganisms. Third, the intestine has an immunological barrier formed e.g. by immunoglobulins and antimicrobial peptides that contribute to pathogen elimination. Finally, it contains cells, such as dendritic cells and intraepithelial lymphocytes, that penetrate or situate in proximity of the IECs and can recognize microbes (Figure 1).

Beneath the IEC layer of the intestine is found the **lamina propria** (LP) layer, a dynamic immune compartment. Effector immune cells in the lamina propria belong to both the lymphoid lineage (T and B cells) as well as **myeloid lineage**. Myeloid lineage cells, such as macrophages (Mfs) and dendritic cells, are key drivers of the innate immune responses within the intestinal immune milieu. Upon physiological stimulation, for instance during infections or diseases, immune cells communicate with each other and with the IECs through a complex network of **cytokines and soluble proteins** ("the common language").[14] In addition, within the LP layer are found lymphoid structures

such as Peyer´s patches, gut-associated lymphoid tissue, mesenteric lymph nodes (in the mesentery), in all of which the priming of adaptive immune responses occurs.[15] (Figure 1)

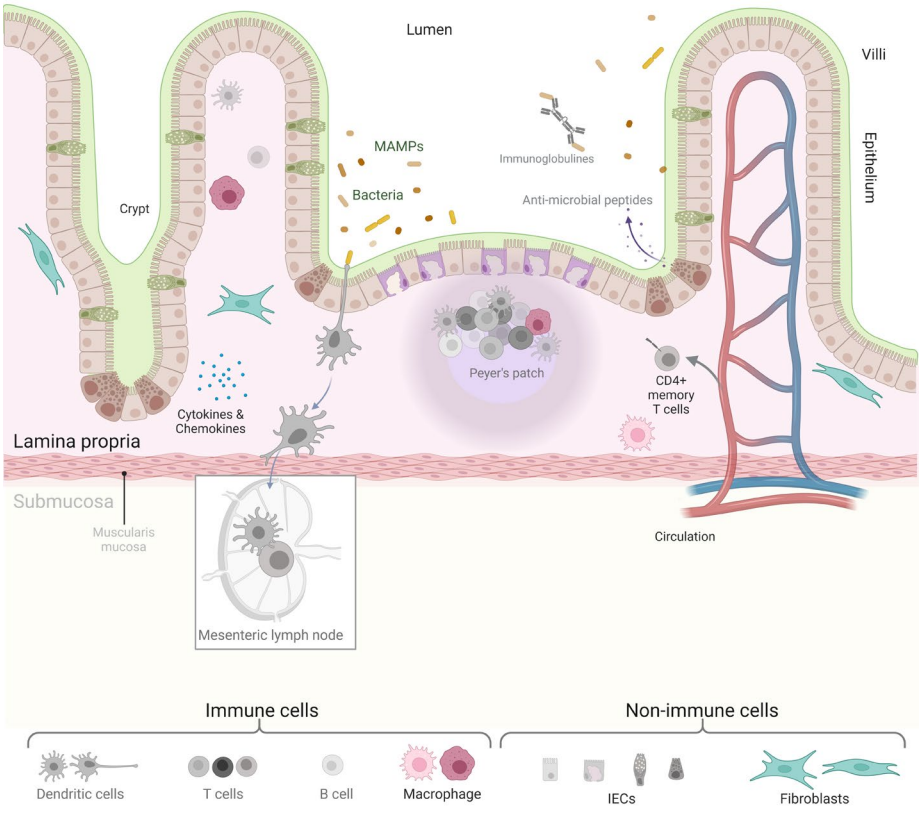

Figure 1. Immuno-anatomy of the healthy human small intestine (ileum) simplified by showing cell and molecules of the intestine. Color greyed cells were not touched upon in this thesis whereas colored cells were studied to different extents.

Immune cells and IECs contribute to proper functioning of the intestine during **health** and **homeostasis**. During health, the intestinal IECs form a barrier to the luminal passage, communicate with the microbiota and actively produce antimicrobial peptides and mucins. Upon facing microbiota, IECs produce mediators (cytokines, chemokines) that signal to the underlying immune cells, or transport pathogenic components.[16] Some underlying immune cells in the LP have as focal function maintaining of the homeostasis by eliminating

dead/apoptotic cells by for instance efferocytosis (macrophages), processing of pathogens and presentation of their antigens (dendritic cells, macrophages).

The human intestine is also a **self-healing** tissue able to repair numerous temporary damages during health.[17] It is able to do so by employing immune-like cells in the stromal compartment which bi-directionally communicate with immune cells to maintain homeostasis.[18] The main cells of the intestinal stromal compartment are fibroblasts, which in different stages and activation phases are named differently (myofibroblasts, trophocytes, telocytes). Fibroblasts contribute to maintenance of homeostasis by bi-directionally communicating with IECs and immune cells (both intraepithelial and LP).[19] However, the fibroblast location within the intestinal mucosa (Figure 1), hence the phenotypical surface marker expression, dictates the function and fate of specific fibroblasts. For instance fibroblasts expressing alfa-smooth muscle actin (α-SMA) are subepithelial fibroblasts that have been associated with the contractile trait of myofibroblasts.[20]

1.2 INFLAMMATORY BOWEL DISEASES

In the onset of several intestinal diseases, there is a switch from physiological homeostasis or health, to an acute and finally **chronic inflammation** of the intestinal mucosa. One group of these diseases is inflammatory bowel disease (IBD). **IBD** is a class of gastrointestinal diseases with the major entitites Crohn´s disease (CD) and Ulcerative colitis (UC). In the next subchapter I divide CD and UC but mainly focus on **CD** for the purpose of this thesis. Both CD and UC for historical reasons have been, and continue to be, studied side by side[21] because they manifest with several similar symptomatology. However, the two diseases are clinically and immunologically/biologically very different.

The number of people with diagnosed IBD between 1990 and 2017 increased from 3.7 million to more than 6.8 million (85.1% increase) in global cases.[22] IBD pathogenesis is very multifaced but the disease is believed to develop by a combination of factors acting in collaboration, such as genetic predisposition, changes in the gut microbiota, **immune system dysfunction** and environmental factors.[23] Regarding the immune system dysfunction, hence chronic inflammation occurring in the intestine of IBD patients, it leads to abnormal immune responses that drive disease pathogenesis and progression.[24]

Therefore a lot of efforts have been made to investigate IBD from an immunological standpoint. In fact, most of the effective treatments (also called biologics) target immune circuits in IBD (e.g. anti-TNF treatment)[25]. Current IBD **treatments** either are limited or function only in a fraction of patients or do not stop the development of complications which drastically affect the patient's quality of life. To add to the complexity and challenges of treatment investigations is that of a notable patient- and immunological-variability. Hence, huge efforts are being made to understand patient subgroups, responses to therapy (responders *vs* non-responders), active disease *vs* remission, and finally why certain complications, such as formation of stenotic tissue, occur and how they can be prevented/treated. Efforts to understand and study the disease more in depth increasingly consider the immunological aspects as key drivers of disease pathogenesis.

1.2.1 DISEASE ACTIVITY IN CD

CD is a chronic disease characterized by alternating periods of active **inflammation and remission** while the disease progresses into more severe complications (Figure 2) for most patients. Despite all the efforts, there is no cure for CD, and the efficacy of treatments varies greatly across patients. Patients with CD suffer from mild to severe symptoms and clinical manifestations, including: diarrhea, fever, fatigue, pain, feces with blood, mouth sores, weight loss, drainage around the anus (fistula), extraintestinal inflammation (multiple organs), kidney stones, anemia, malabsorbtion.[26,27] For most patients the disease progresses into complications, that can arise shortly or long after diagnosis[28], such as strictures, obstructions, fistulas, vitamin and mineral deficiencies and/or even cancer. CD complications together with symptoms drastically impact the everyday quality of life of patients and some of these complications also require major surgery with long recovery times and surgery-related complications.

A patient with CD is assessed to have **active disease** when a clinician establishes the activity via a series of questionnaires and measurements. To establish with certainly if a patient has active disease (inflammation), one of the following is needed: endoscopy (or gastroscopy if the upper GI is affected), magnetic resonance imaging, or ultrasound. However, sometimes these can be considered too invasive. Following, other assessments are made such as of biochemical markers and/or symptoms. For this thesis work, the Harvey-Bradshaw Index (HBI) (Paper I and II) and Crohn's Disease Endoscopic Index

of Severity (CDEIS), C-reactive protein (CRP), calprotectin (Paper III) were used.

CDEIS is an index used to define activity focused on endoscopic activity in patients with CD. Patients having CDEIS ≥5 are considered to have ongoing active CD. The CDEIS evaluates the rectum, sigmoid and left colon, traverse colon, right colon, and ileum in the GIT of patients with CD.[29] For each colonic segment, the presence of various types of ulcerations as well as stenoses are measured. These measurements are added and the total index can range from 0 to 44, where the higher the CDEIS number the more severe activity the patient has.[30,29] The practice of CDEIS, although widely used in research, requires further investigations to define operating terms[30] and is normally performed by a gastroenterologist. Patients having a **HBI** > 4 are regarded as having ongoing active CD. HBI is a questionnaire that is composed by nearly half of the questions that can be regarded patient subjective (pain, diarrhea/number of liquid stools, general wellbeing) and half of them as more objective (abdominal mass, complications).[31] However, it is easily obtainable and widely used in research[32], as it does not require performing an endoscopy or biochemical tests. This was also the reason that it was established just before the surgery, given that our CD cohort was a surgical cohort (Paper I and II).

Besides questionnaire- and endoscopy-based indexes, there are also a few commonly used biochemical markers for defining the disease activity in patients with CD. **CRP**, an acute phase protein used as a serological marker and **calprotectin,** analyzed in feces**,** are good measurements of either colonic CD but less reflective for ileal involvement (calprotectin) or better for ileal disease than colonic (CRP).[33,34] In the intestine, calprotectin is released by activated neutrophils which recruitment in increased during CD inflammation. Of note, although the measures above are commonly used, further new biomarkers, such as myeloperoxidase (MPO), human neutrophil lipocalin (HNL), and eosinophil-derived neurotoxin (EDN) are being compared with the widely used calprotectin marker.[35] In addition, indexes and scores such as the simplified magnetic resonance enterography index and ultrasonographic score are under discussion for defining the activity in ileal CD.[36,37] The ideal scenario would include the use of scores, indexes and markers that are specific not only for CD in general terms but that can stratify CD in subgroups such as ileal, colonic, ileocolonic. Since it is frequently not valuable to use a single marker, score, or index to define the disease status or patient subgroup, a combination of markers, scores, and indexes is progressively gaining wider use in research in order to confirm the occurrence of real activity and flares.

1.2.2 COMPLICATIONS IN CD

Complications characterizing CD can arise in patients at any stage of the disease and can manifest locally in the GIT or distantly in other organs. Furthermore, specific factors such as ileal involvement and perianal disease[28], as well as male sex[38], have been associated with incidence of complications in CD. In addition, given that inflammation in CD can be transmural in nature, meaning a loss of the 5-layer structure of the intestine, some of the common complications occurring locally are strictures (stenosis) and fistulas. Strictures in CD are clinically defined as either inflammatory or fibrotic strictures. This clinical classification does not reflect the biology of the stricture (the underlying processes) but is rather a coarse naming, by clinicians, to indicate if the stricture has responded to anti-inflammatory drugs (that they name inflammatory stricture) or not (that they name fibrotic stricture). However, biologically strictures are very complex and not-well defined, and the activity of both fibrotic processes and inflammatory processes (immune cells involvement) contribute to the formation of the stricture *a priori*. Strictures are the end stage and are formed by fibrotic build up, hence narrowing of the GIT lumen, and it is predicted that circa 70% of patients with CD will develop strictures 10 years after diagnosis.[39] Current treatment for already developed strictures include surgery or dilatation by an inflated balloon. Regarding pre-stricturating status, some biomarkers are proposed to predict which patients with CD are more at high risk to develop a strictures, such as nucleotide-binding oligomerization-containing protein 2 (*NOD2)* variants.[40] Serological biomarkers have been proposed for differentiation of stricturating behaviors, such as those for collagen turnover.[41] However, there is no way to prevent the formation of strictures per se and all the consequences that come from it. Complications in CD, related to fibrosis, make it difficult to study CD disease using animal models (e.g. TNBS, DSS-induced colitis, IL-10$^{-/-}$ mice) where the disease develops rapidly. Despite many animal models being useful in studying candidate treatments, inflammatory processes, wound healing mechanisms and immune cell dynamics, they cannot fully reflect the human CD complexity including the transmural inflammation and penetrating segments or the heterogeneity of patients.[42,43]

1.2.3 DIFFERENCES BETWEEN CD AND UC

Ulcerative colitis (**UC**) also falls within the IBD categorization. Papers I and II cover a few aspects related to UC hence it is important to distinguish UC from CD. Like CD, UC is a disease characterized by chronic intestinal inflammation but while CD can occur anywhere in the GIT, UC is limited to the colon (Figure 2). Unlike CD, **inflammation in UC is continuous**. Furthermore, inflammatory lesions in patients with UC are typically proctitis (i.e. in distal colon) or left-sided colitis, but in about 20% of the cases affects the full colon (defined as pancolitis or extensive colitis).[44] The human colon has a thickened mucus layer and a higher load of microbiota compared to small intestine.[15] By using proteomics on mucus of UC patients it has recently been shown that structural components of the colonic mucus in UC are decreased in both inflamed and non-inflamed areas. IEC goblet cell secretory responses to microbes occurs regardless of inflammation suggesting this to be an event happening on disease onset.[45]

Regarding innate immunobiological aspects, studies have shown important differences between UC and CD both in the intestinal tissue and blood.[46,47,48] For instance blood from patients with active CD separated from active UC by an increased population of **IL-1β**$^{+}$ dendritic cells, IL-1β^{+} monocytes, and fewer group 1 innate lymphoid cells.[46] In addition, genetic studies have shown variants, such as ***NOD2***, to be protective for UC but to increase the risk for CD.[47,49] NOD2 is an intracellular pattern-recognition receptor (PRR) and plays a role in immune responses by recognizing specific structures in the bacterial cell wall named muramyl dipeptide[50]. Differences in the role of *NOD2* in CD and UC pathogenesis suggest specific and distinguished innate immune responses in these two diseases.

Although CD and UC present overlapping symptoms (abdominal pain, blood in feces, weight loss), separate scores are used to define disease activity in these two diseases. One of the disease activity scores used most in research and clinics for UC is the **Mayo score**,[51] which was also used in papers I and II of my thesis. The Mayo score includes questions on the frequency of bowel movements, rectal bleeding, endoscopic findings and the global assessment by the gastroenterologist.[51] There is the Full Mayo score and a simplified version, the Partial Mayo score[52], which excludes for instance the endoscopy part. Both Full and Partial Mayo scores are use in research. While a number of clinical trials assessing therapeutic drugs use the Mayo score, there is still an ongoing

debate on specific cut offs of the score for remission, clinical significance and validity.[53,54]

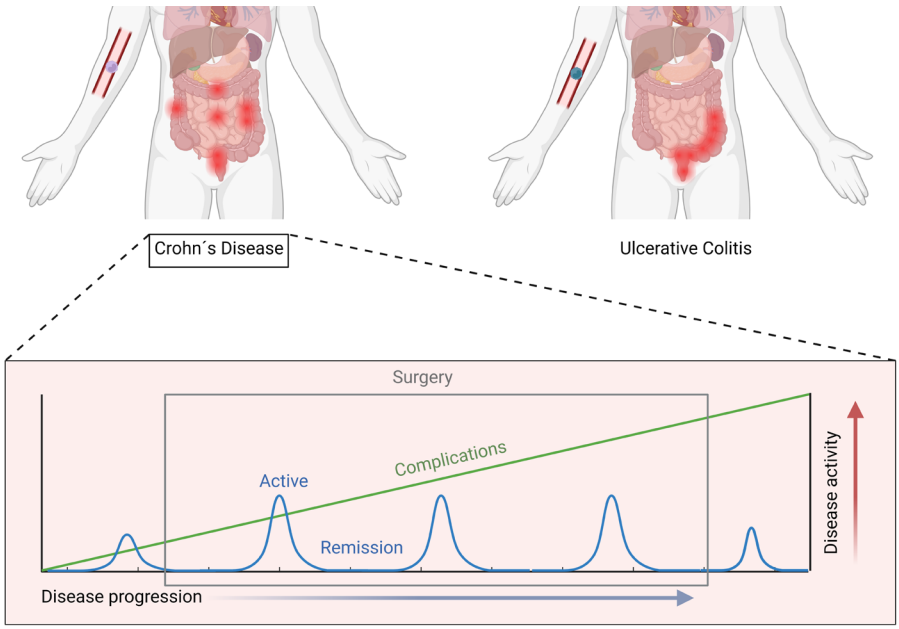

Figure 2: Disease course in CD with longitudinal dynamics (bottom) and visual differences in disease localizations between CD and UC.

1.2.4 CD AND INTESTINAL INNATE IMMUNITY

CD is mainly characterized by chronic intestinal inflammation, but the discontinuous, transmural inflammation can arise anywhere in the gastrointestinal tract (Figure 2). However, inflammation in approximately 80% of cases affects the distal part of the small intestine (CD ileitis, CD ileocolitis) and in approximately 30% of cases affects the colon (CD colitis).[44] Patients with CD have an impaired intestinal barrier function that, by letting passage of food and microbial components (Figure 2), can shift different protective innate immune mechanisms of the intestine.[55] Mechanisms related to intestinal barrier function include **alterations of intestinal PRRs**, compromised mucus layer, increased permeability, and changes in antimicrobial peptides production. As a consequence the mucosal epithelium is breached creating an easy way for bacteria and food particles to pass into deeper layers of the mucosa.[56] For instance, IECs such as goblet cells, which are responsible for mucus

production, are shown to have a higher differentiation ratio and an association inflammation in CD that was not shown for UC.[57] Below the epithelium, the intestine of patients with CD have a stretched LP layer. The enlargement of the LP occurs due to an increase in recruitment of immune cells during the disease but especially during flares (Figure 2).

Enlargement of the lamina propria occurs upon the epithelium breach where there is a continuous passage, mainly of bacteria, from the lumen to the lamina propria. The bacterial load causes a local acute inflammation that with prolonged exposure drives expansion of the LP layer and the chronic inflammation characteristic of CD. The innate immune cells are key performers and the first line of defense in fighting particle- and bacteria-induced inflammation. The inflammation in active CD patients is chronic, with repeated periods of flares, with continuous recruitment/replenishment of the innate immune cells. During health and homeostasis, both IECs and innate immune cells contain PRRs and signaling receptors able to recognize pathogens while maintaining tolerance to commensals. During CD in contrast, the role of these receptors, such as Nucleotide-binding oligomerization-Like Receptors (NLRs) and Toll-Like Receptors (TLRs) are increased in activity or show enhanced responses contributing to the ongoing inflammation.[58,59,60] However, due to the breach of the epithelium during CD and passage of commensal microbiota, the innate immune cells will also react to commensals. Hence, this sharp division (recognition of pathogens *vs* commensals) is not clear for CD intestinal cells which can also react abnormally to commensal microbiota during disease progression.

1.3 INNATE IMMUNE CELL TYPES AND ROLE IN CD

The intestinal innate immune system cells include macrophages, monocytes, dendritic cells, neutrophils, eosinophils, basophils, mast cells, innate lymphoid cells, and natural killer cells. Innate immune cells are involved in the **pathogenesis of CD** and, in contrast from the adaptive immunity, they have in common the ability to rapidly induce non-specific responses to any danger affecting the intestine of CD. For the limited purpose of this thesis, I will give an overview to macrophages, monocytes, dendritic cells and neutrophils with more details below about macrophages.

1.3.1 MONONUCLEAR PHAGOCYTES

The mononuclear phagocytes (MNPs) comprise three types of innate immune cells: **macrophages, monocytes** and **dendritic cells**. These three cell types, although distinct cell types, have overlapping functions and progenitors (differentiating from the same cells) that groups them into MNPs. Regarding the intestinal MNPs they are of **prenatal** origin (fetal liver, yolk sac) or **postnatal** origin (bone marrow). The MNPs of prenatal origin develop during embryogenesis in the tissue.[61,62,63] In case of the postnatal derivation, MNPs descend from a common myeloid progenitor that differentiates into blood monocytes and blood dendritic cells which later on will turn into intestinal macrophages and intestinal dendritic cells (Figure 3).[64,65,66] According to the presence and quantity of surface markers CD14 and CD16 circulating adult human **blood monocytes** are categorized into **classical** ($CD14^{++}CD16^{-}$), **intermediate** ($CD14^{++}CD16^{+}$) and **non-classical** ($CD14^{+}CD16^{++}$) monocytes respectively.[67,12] Hence, the majority of blood monocytes are $CD14^{high}$. It has been widely shown that patients with CD are characterized by high levels of monocytes in the blood (monocytosis) that has also been proposed as a potential biomarker to predict disease severity.[68,69]

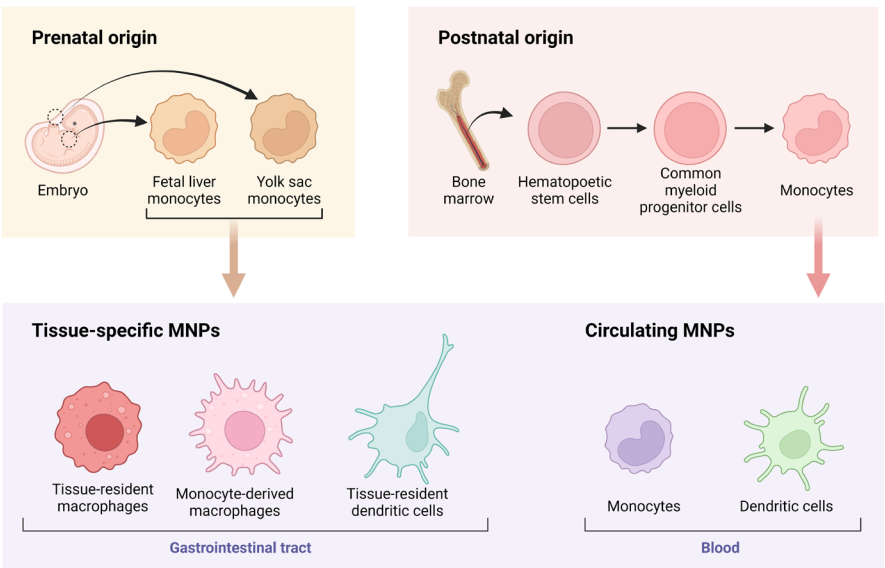

Figure 3. Progenitors and derivation of MNPs in the human intestine.

Dendritic cells and macrophages can originate from the human bone marrow or locally while monocytes originate in the bone marrow. Monocytes are present in the blood circulation and continuously waiting for signals from different organs. During intestinal inflammation monocytes migrate from the circulation into the intestine where they give rise and differentiate into monocyte-like/immature macrophages or monocyte-derived dendritic cells. This differentiation is directed by cytokines and growth factors such as macrophage colony-stimulating factor (M-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF).[70] Monocyte-derived dendritic cells (1) have the same myeloid progenitor as the bone marrow common dendritic progenitor (Figure 3). Common dendritic progenitor cells give rise to plasmacytoid dendritic cells (2) as well as conventional dendritic cells (3). All three types of dendritic cells can be found in the intestinal LP, although conventional dendritic cells, defined into subsets cDC1 and cDC2, are the most effective antigen-presenting cells.[71,72] Dendritic cells sample bacteria and bacteria particles by both direct contact with certain IECs as well as by piercing the IEC monolayer to be in direct contact with the GIT lumen (Figure 4).[73] Their function as MHC class II **antigen-presenting cells** allows guarding of local tissue, for example upon encountering of an antigen by migrating to lymph nodes to activate antigen-specific naïve T cells. Hence, maintaining tolerance to commensal bacteria and ensuring homeostasis. CD is characterized by loss of tolerance to commensal bacteria[74], but it is not entirely clear to what extent dendritic cells play a role. However, some populations of conventional dendritic cells are decreased in number during CD and it has been supposed that this is due to migration or loss due to inflammation[75,76] (Figure 4).

Macrophages (Mfs) are the main cells investigated in this thesis work. A considerable amount of space is allocated to these cells in the subchapters below where I subdivide the understanding of the field based on the research performed on tissue, primary cells, or cell lines with referral to CD. I write about Mfs already here to put these cells into context with the other MNPs and their overall involvement in innate immunity related to CD. In health macrophages are located in different GIT layers including the LP layer, muscularis mucosa and submucosa**. LP layer macrophages** exhibit remarkable heterogeneity, some are long-lived cells and support the intestinal functions that allow for instance maintenance of the tissue as well as healing. Macrophage functions include, among others, phagocytosis, efferocytosis,

immune modulation and repair of tissue damage. These innate functions go hand in hand with specific surface markers macrophage subsets express and proteins they release to interact with adjacent and distant cells shaping and being shaped by the intestinal microenvironment. During inflammation in CD, monocytes are recruited to the site of infection by means of for example MCP-1 (monocyte chemoattractant protein 1) by expressing its receptor CCR2 (C-C motif chemokine receptor 2)[77,78,79,80]. Consequently, numerous $CD14^+$ macrophages resembling monocytes dominate and accumulate in the inflamed mucosa of patients with CD (Figure 4).[81] There is increasing evidence that suggests deficiencies in macrophage differentiation, maturation, activation and an underlying link between defects in the resolution of intestinal inflammation in patients with CD.[82]

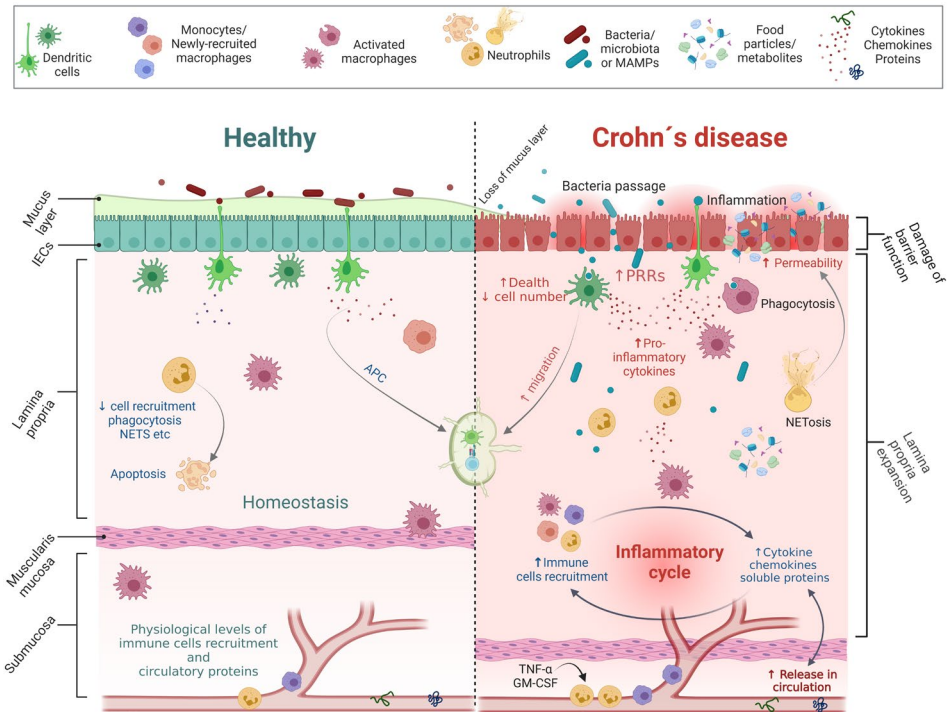

Figure 4. Overall schematic overview of immunological alterations in the intestine during CD focused on innate immune cells. Detailed emphasis is placed in CD for the purpose of this thesis with healthy condition as reference.

1.3.2 INTESTINAL MUCOSA MACROPHAGES

1.3.2.1 FUNCTIONS

The intestine is an organ exposed to constant heavy loads to dietary antigens, pathogens and commensals or their components, namely microbe-associated molecular patterns (MAMPs).[83] These loads of microbes and MAMPs can cross the IEC barrier by various routes reaching deeper layers and encountering immune cells such as Mfs. Intestinal Mfs are located throughout the GIT mucosa with a large proportion of them situated in the LP in proximity to the epithelium.[84] Intestinal Mfs are expert multitaskers of the human body (Figure 5). They functionally impact GIT tissue and its immune homeostasis and health[85] by:

A. Triggering non-specific immune responses (and consequently produce a variety of cytokines and soluble proteins).[86]
B. Being bacterial scavengers: performing phagocytosis (killing of pathogens) e.g. during infections[87] to restore homeostasis.
C. Recognizing bacterial products through their PRRs.
D. Being antigen-presenting cells and evoking mainly type 1 T helper cell signaling.[88] Being sessile cells, they perform this locally rather than migrating to lymph nodes (like dendritic cells do).
E. Performing efferocytosis (elimination of dead and apoptotic cells) which allows clearance of the tissue.[89,90]
F. In case of an injury contributing to the resolution of inflammation and tissue healing.[91]

The first three functions (Figure 5) and the Mfs performing them are referred to as pro-inflammatory. In contrast to the other functions, the last two (Figure 5) are more pro-resolution or wound healing functions that in the context of Mfs are most often referred to as anti-inflammatory. The functions performed above are not universal to all Mfs, but rather specific Mfs subsets perform either restricted functions or the same function to different extent. Function-wise, Mfs are key immune contributors to CD pathophysiology, although the onset of CD is not attributed to one single immune cell type, given the complexity of the tissue. As mentioned above in relation to CD, the number of pro-inflammatory $CD14^+$ macrophages are increased during CD to fight the ongoing inflammation triggered by MAMPs on pathogens or commensals. As a consequence, the function of these Mfs in CD will predominantly be to contribute to the pro-inflammatory disease environment by producing

increased numbers of inflammatory cytokines, such as TNFα, IL-1β, IL-23 and IL-6, that contribute to recruitment of more immune cells, the disease microenvironment and chronic inflammation characterizing patients with CD.[92,93,94] While some functions, such as those with pro-inflammatory involvement, are widely and historically studied for Mfs in CD, we lack knowledge for other functions such as efferocytosis (addressed in Paper III).

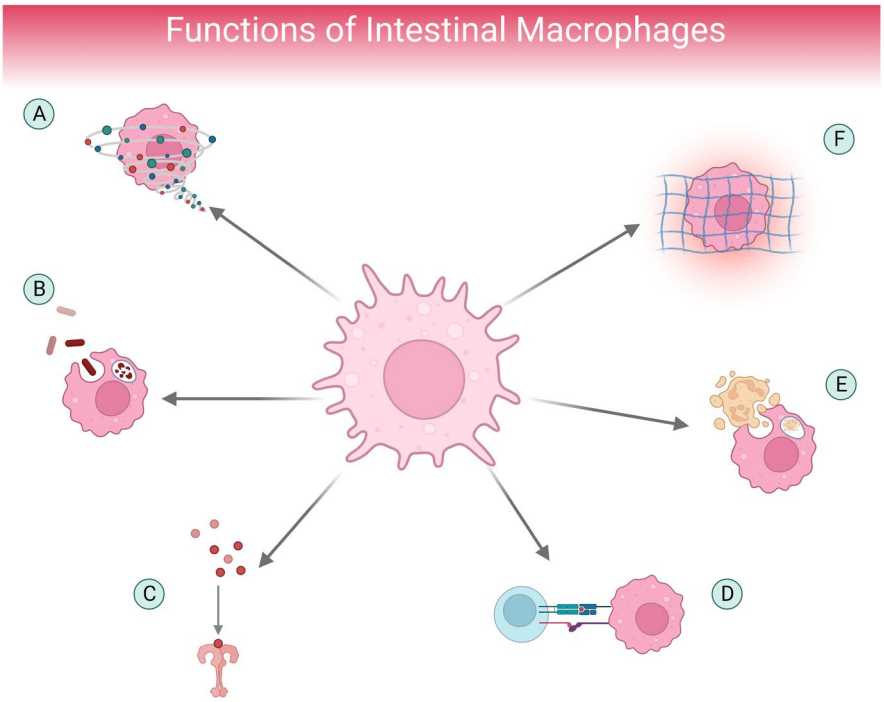

Figure 5: Summary of intestinal macrophage functions. (A)Cytokines/inflammatory proteins secretion, (B)Phagocytosis, (C)Recognition of MAMPs, (D)Antigen presentation, (E)Efferocytosis (F) Tissue healing.

1.3.2.2 IDENTIFICATION AND SUBSETS

Conventionally, and even at present in immunology books[95], macrophages are categorized into classically activated/pro-inflammatory (M1) or alternatively activated/anti-inflammatory (M2). However, this is an outdated concept as it does not capture the real dynamics of Mfs, and it is widely shown in the last decade's research that when it comes to Mf subsets, there is more to just this basic classification in the GIT. What are the subsets currently agreed on, where do they come from and how do we identify them?

Tissue Mfs originate from both embryogenic Mf precursors and circulating blood monocytes. Bone marrow derived classical monocytes circulate in the blood for 1 day[96] before arriving at the GIT where they acquire a specific Mf phenotype guided by the local microenvironment.[78,97] Monocytes can then leave tissues through the lymphatic system or undergo apoptosis/clearance in the tissue itself.[61,98,99] On the other hand, the tissue-resident Mfs of embryogenic origin reside in the tissue before birth and have the capacity to self-renew.[61,62,63] Intestinal Mfs have in common with other MNPs many recognized cell surface markers that are needed for their identification in the tissue. For instance, the markers CD11b and CD11c are surface markers expressed both by some Mfs and some subsets of dendritic cells (Table 1).[100] These overlaps of common markers have traditionally hampered the exact identification of Mf subsets.[85] However, the elucidation of existing markers and identification of new markers, together with the evolving methods of marker co-detection (e.g. multicolor flow cytometry) are continuously advancing our understanding of specific subpopulations and their roles in health and disease. A second reason for uncertainties in Mf research has also been the knowledge transfer from animal (e.g mice) to human studies. Animal studies were widely used for fate-mapping, and a number of Mf markers (such as TREM-1) are conserved between species, however the differences drastically outweigh the commonalities.[42] Finally, identification of Mf subsets has developed side-by-side with the specific localization of Mf, that at this point has gone beyond within GIT differences (e.g. small *vs* large intestine) and more and more studies are associating Mf phenotypes and function with specific localizations. For instance, Mf subsets with high antigen-presenting and phagocytic capacity localize in proximity to the epithelium which is the most strategic position to encounter bacteria.[101,42,102]

I will next describe the mucosa Mf subsets functionally identified in the intestine in health and CD. Based on surface marker expression of HLA-DR, CD14, CD11c and CD11b there are currently four described subsets of intestinal Mfs in the **healthy human small intestine**: **Mf1** ($CD14^+CD11c^+HLA\text{-}DR^{int}$), **Mf2** ($CD14^+CD11c^+HLA\text{-}DR^{hi}$), **Mf3** ($CD14^+CD11c^-CD11b^-$) and **Mf4** ($CD14^{hi}CD11c^-CD11b^+$).[79,65] Mf1 and Mf2 were shown to be phenotypically similar to circulating monocytes and are also called for convenience **immature Mfs**, while Mf3 and Mf4 were shown to be phenotypically similar to **mature Mfs**.[79] By using advanced single cell RNA sequencing, $CD11b^+$ Mfs (likely Mf4), named also self-maintaining Mfs, were shown to not be replenished by monocytes and their depletion leads to the disruption of essential homeostatic functions, resulting for example in

morphological defects in intestinal vascular and enteric systems as wells reduced/lost mobility.[103] In addition, all four Mf subsets were shown to be transcriptionally different from monocytes and dendritic cells,[79] overcoming the issue of surface marker similarity abovementioned for MNPs. Functionally, compared to monocytes the four Mfs subset are less responsive to treatment with inflammation (cytokine) inducing MAMPs (e.g. LPS, flagellin), demonstrating that despite similarities with their monocyte precursors, Mfs are shaped by the local microenvironment.[79] However, all four Mf subsets were able to perform endocytosis in the tissue. The first three Mf subsets have more endocytic capacity compared to Mf4 and they have similar capacity to monocytes to perform endocytosis.[79]

Given the strong indications that Mfs are involved in CD pathophysiology, efforts are under way in understanding their behavior in CD *per se* and in their relationship with current treatments[104]. Consistent with the data above for healthy human mucosa, in **CD colonic mucosa** there are reported Mf subsets CD11c^{high}, CD11c^{dim} and CD11c^{-} that share characteristics with Mf1, Mf2, Mf3.[105] Bernardo *et al.* showed that only **CD11c^{high}** pro-inflammatory newly-arrived cells (probably equivalent to Mf1) were increased in the inflamed colon of CD.[105] Of note, although no transcriptional analysis was performed in this study to functionally distinguish the subsets identified with surface markers, the frequency of CD11c^{-} Mfs (likely Mf3) increased when exposed to conditioned media from healthy intestinal mucosa indicating that this Mfs subset is most likely more similar to Mf2 rather than Mf4.[105,65] Mf subsets have also been defined by their expression of intracellular PRRs. Combined microscopy and single cell analysis has shown that Mfs in the colon of CD are defined by the NLRP3 and are spatially co-localized with neutrophil infiltrations.[42,106] This is quite significant in CD as missense mutations in Caspase activation and recruitment domain family member 8 (CARD8), a protein which inhibits the NLRP3 inflammasome, leads to hyperresponsive monocytes in CD and patients with a CARD8 mutation were resistant to anti-TNF agents.[42,107] Inflammasomes are discussed in the following chapters and investigated in Paper I of this thesis.

In summary, Mfs in the intestinal mucosa are divided into four functionally and phenotypically different subsets, ranging from pro-inflammatory newly-arrived monocyte-like cells to tolerogenic tissue-resident cells.[79,105,65] The four subsets are a continuum ("waterfall"), differentiate in a stepwise manner, where the first three are differentiated from each other, Mf1 into Mf2 and Mf2 into Mf3, and the Mf4 subset is purely from embryogenic origin residing in the

GIT since birth but shares communalities with Mf3.[79,105,65] In CD colon, these processes are impaired and there is an increase of monocyte-derived cells, consistent with the continuous recruitment of monocytes from the blood flow into the intestine, e.g. through CCR2, to fight the passage of bacteria/particles.[79,105,65] Up to this stage in literature, to my knowledge, the Mf1-Mf4 characterization had not been studied for **small intestine in CD** (including ileum) – a gap which was addressed in Paper II of my thesis.

Table 1. Outline of Mfs markers used in this thesis, their functions, and role in CD.

Marker (type, synonym)	**Cell/s** expressing the marker. (1) Primary/High (2) Secondary/Low expressed	**Function of Mfs** that are +/high for the marker	**Evidence/role in CD**	**Ligands**	**Ref**
CD14 (glycolipid-anchored membrane glycoprotein)	(1)Mfs, Monocytes, Dendritic cells, (2)Neutrophils	High cytokine production, Antigen-presenting	Increased $CD14^{+}$ Mfs numbers and cytokine production during CD	Lipopolysaccharide (LPS), Lipopolysaccharide binding protein (LBP)	93, 88, 94
HLA-DR (αβ heterodimer/ MHC class II cell surface receptor)	(1)Mfs, Monocytes, Dendritic cells, B cells	Antigen-presenting	High proportion of $HLA\text{-}DR^{dim}$ (resembling classical monocytes) in CD inflamed vs uninflamed mucosa.	Peptides (ligand for T cell receptor)	81
CD11c ((integrin alpha X chain (ITGAX) protein, type I transmembrane protein))	(1)Mfs, Monocytes, Dendritic cells, Neutrophils	Endocytosis, Present in Mf1-Mf2 (Mf1-Mf4 classification)	$CD11c^{high}$ pro-inflammatory monocyte-like Mfs are increased in CD (inflamed colon).	Fibrinogen, complement fragment (iC3b), ICAM-1	105,79
CD206 ((C-type lectin, macrophage mannose receptor (MMR))	(1)Dendritic cells, (2)Mf, Endothelial cells	Wound healing, Efferocytosis, Mfs maturation	Mucosal healing during anti-TNF treatment. Differentiates newly-recruited from tissue-resident Mfs.	Mannosylated ligands (i.e. present on microbes)	91, 108, 109, 110

CD11b ((Integrin alpha M (ITGAM))	(1)Mfs, Monocytes, Granulocytes, Natural killer cells (2)T cells, B cells	Efferocytosis, Chemotaxis, Adhesion (endothelium, extracellular matrix), Identify Mf4 subsets	Activation of CD11b (in animal models of intestinal inflammation) increased chemotaxis of leukocytes and decreased inflammation in inflamed mucosa.	Extracellular matrix proteins, complement fragment (iC3b), ICAM-1	111, 112, 113, 79
CD209 (C-type lectin receptor, dendritic cell specific ICAM3-grabbing non-integrin)	(1)Dendritic cells, (2)Mfs	Antigen presenting, Phagocytosis, Identify Mf3 and Mf4 subsets	Mfs subset induces higher potential of Th1 responses in CD inflamed compared to controls, and light potential to induce Th17 that was enhanced by commensals only for CD.	ICAM-3, HIV-1 envelope glycoprotein gp120	88
CD36 (lipid receptor, highly glycosylated class B scavenger receptor)	(1)Mfs, (2)Monocytes, Dendritic cells, Neutrophils	Efferocytosis, lipid metabolism, Induction of TGFβ1 synthesis.	Involved in response against *Mycobacterium avium subsp. paratuberculosis* (in THP-1 cells) which is associated with CD.	Lipids, high-density lipoproteins	114,115 116,117 118
CD163 (high affinity scavenger receptor cysteine rich family type B)	(1)Mfs, Monocytes, (2)Dendritic cells	Th1 and Th2 responses.	Increased soluble (circulating) CD163, as a marker of macrophage activation, in active CD and that was decreased by antiTNF therapy. Overexpression of mucosal CD163 in active CD and not affected by other therapies.	Gram-positive and gram-negative bacteria	119, 120, 121
CD80 (B7-1, type I membrane glycoprotein, immunoglobulin)	(1)Activated Dendritic cells, B cells, (2)Mfs	Antigen presenting, proinflammatory Mfs marker.	CD80 upregulated in CD inflamed mucosa where $CD80^{+}$ cells were also $CD14^{+}$. No increase is CD monocytes.	CD28 and CTLA-4	122, 123
CD64 (Fc receptor FcγRI)	(1)Macrophages, Monocytes, Neutrophils Eosinophils	Distinguishes Mfs from dendritic cells together with CD163. Phagocytosis and ADCC	Increased expression and correlation with disease activity in CD.	Immunoglobulin G1, G3 and G4	124,125 ,126, 65,127, 128

Mfs, Macrophages; LPS, Lipopolysaccharide; CD, Crohn´s disease; CD(number), cluster of differentiation; Th, T helper cells; HLA-DR, Human Leukocyte Antigen-DR isotype; MHC, Major Histocompatibility Complex; CD11b named also macrophage-1 antigen (Mac-1) or complement receptor 3 (CR3). ICAM, Ig superfamily cell adhesion molecule. CD36 also known as platelet glycoprotein 4, fatty acid translocase (FAT), scavenger receptor class B member 3 (SCARB3), and glycoproteins 88 (GP88), IIIb (GPIIIB), or IV

(GPIV), is expressed in other non-immune cells as well: platelets, erythrocytes, adipocytes, muscle, epithelial cells, spleen cells, skin endothelial cells. THP-1, macrophage cell line; CTLA-4, Cytotoxic T-lymphocyte-associated protein 4. CD163 is traditionally regarded a mature macrophage marker although there is evidence suggesting showing that it is a non-specific marker for immature/mature Mfs[121]. ADCC, Antibody-dependent cellular cytotoxicity; IFNγ, interferon γ; G-CSF, granulocyte colony-stimulating factor. CD64 is mainly expressed by MNPs, resting neutrophils scarcely express CD64, although upon IFNγ and G-CSF acting on the neutrophil precursors in the bone marrow the expression increases drastically.

1.3.2.3 MACROPHAGE-MICROBIOTA INTERACTIONS

The presence of the intestinal microbiota and/or its products influence Mf dynamics in the GIT and this is proven by studies using germ-free mice where monocyte recruitment in the GIT is diminished given the lack of microbial signals.[42,129] Function-wise, Mfs are the main phagocytes and they can also perform antigen presentation and support mucosal immune defenses in the GIT, hence it is not surprising that they are highly functioning in a microbe rich GIT environment, although it might be surprising that during health they have tolerance to microbes without producing excessive pro-inflammatory signals.[85] However, during intestinal injury, Mfs can become hyperactivated when sensing microbes through their repertoire of PRRs. For instance, during intestinal injury, certain microbiota constituents induce the pro-inflammatory cytokine IL-1β via the NLRP3 inflammasome[82,130] which are a crucial cytokine and PRR of immature (newly-recruited) Mfs in the intestine respectively.[131] In addition, microbiota-induced IL-1β is needed for T helper 17 cell responses in the intestine[132] which increase in CD and contribute to disease pathogenesis and progression[133].

Microbial metabolites, as a reflection of microbial activity, also impact the intestinal immune cells, including Mfs, to shape the local microenvironment dynamics.[134] It is shown that, throughout the course of CD, the intestinal microbiota are stable[135,136] but temporal alterability has been reported[21]. Regarding intestinal metabolites, there is robust evidence on the differences between CD and healthy in the metabolomic profiles[137], however it is not clear how metabolomic profiles change throughout the course of the disease since microbiota composition does not linearly reflect microbial activity. Only a a limited number of studies have investigated the effects of bulk fecal metabolites from IBD patients on Mf function and phenotype.[138,139] Priming of Mfs with fecal metabolites from patients with CD, compared to healthy fecal metabolites, reduce Mf bactericidal activity, which is suggestive of a role of secreted metabolites in innate immune functions related to CD pathophysiology.[138] Interestingly, microbiota activity has been investigated in

DSS-colitis and the effects of treatment with an active component of ginger on the microbiota activity showed immediate changes of microbiota and improvement of an *in vitro* anti-inflammatory Mf activity.[140] There is evidence that microbiota and their activity (metabolites) change during CD and that Mfs can detect these changes, being innate immune cells that can signal a vast type of ligands. The impaired or enhanced activity of specific intestinal Mf subsets by microbial-derived factors can be a potential way to measure the targeted effects during microbiota-related candidate treatments.

1.3.2.4 *IN VITRO* MONOCYTE-DERIVED M1 AND M2 MACROPHAGES

I used the *in vitro* model of monocyte-derived M2 macrophages (M2MQ) in Paper III of my thesis. Thus, it is important to spend a few words in defining and describing the M1MQ and M2MQ concepts. Given that the abundance of Mfs, especially the tissue-resident (intestinal) Mfs, is relatively low in humans,[141] it makes it challenging to isolate sufficient numbers of these cells to perform functional studies both in healthy and IBD[65]. Hence, for pre-clinical research the use of the *in vitro* models of polarized macrophages (M1MQ and M2MQ) from blood monocytes is commonly accepted. In this context monocytes for this reason most often are referred to as M0 (Figure 6). Polarization *in vitro* is performed with the help of growth factors and cytokines (Figure 6) that educate Mfs and is a widely used method for functional studies. In addition, monocytes comprise 2-10% of immune cells in blood, and these relatively high numbers[142] make monocytes easily obtainable to polarize into high number of Mfs. Of note, the *in vitro* M1MQ and M2MQ classification is not to be confused with the abovementioned traditional M1 classical and M2 alternatively activated Mfs classification for tissue macrophages (still found in immunology books).[143] However, to my knowledge, the M1MQ/M2MQ usage and naming started from this concept as the aim was to mimic the tissue M1/M2 stratification with strong pro-inflammatory properties (M1) and the other with strong anti-inflammatory properties (M2). Although, in the tissue itself to date the classification of Mfs (Mf1-Mf4), as explained above has more "*in betweens*" (more than one subset of pro- and anti-inflammatory cells), the M1MQ and M2MQ *in vitro* polarization has nevertheless remained the main alternative used to mimic tissue Mfs.

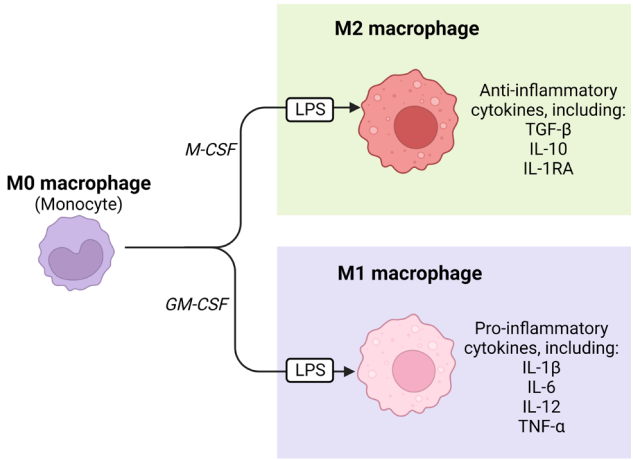

Figure 6: Polarization of blood monocytes, often referred to as M0, into M1 macrophages and M2 macrophages. M-CSF, macrophage-colony stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; LPS, lipopolysaccharide; TGF-β, Transforming growth factor beta; IL, Interleukin; TNF, Tumor Necrosis Factor.

Box 1 – *"This or that"*

Throughout macrophage-related literature, research and lay-language, the observant reader will notice that several names are used to describe the same cells in the GIT. This is not uncommon in immunology as the field progresses, and more is known about surface protein markers and function defining a cell subset, ontogeny and progenitors of immune cells, older names stay, get passed on and sometimes mixed up. This box will explain what the synonyms refer to make it understandable why they are used interchangeably in literature.

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Pro-inflammatory macrophages: refers to the type of cytokines/proteins these macrophages produce and hence the function and contribution they have in the GIT in health and disease. For instance, macrophages produce IL-1β which is known to be a potent pro-inflammatory cytokine.

- *Monocyte-derived macrophages:* refers to the fact that macrophages are derived from circulating monocytes. Monocytes upon entering the intestinal environment encounter signals that polarize them into macrophages.
- *Newly-recruited macrophages:* refers to monocytes as newly recruited, or just arrived, in the intestine but that quickly are polarized into macrophages.
- *Monocyte-like macrophages:* Refers to the fact that macrophages express common surface (recognition) markers that makes it clear that they are derived from monocytes, hence they are monocytes-like.

- *Immature macrophages:* implies that there is a mature type of macrophages. The mature type is, as explained above, the resident macrophages that reside in the GIT since birth or are matured in a *"waterfall"* from monocytes recruited in the GIT. Hence, immature macrophages are macrophages that have not matured to this stage.

_____

Anti-inflammatory macrophages: refers to the type of cytokines/proteins these macrophages produce and hence the function and contribution they have in the GIT in health and disease. For instance, production of IL-10 which is known to be an anti-inflammatory cytokine.

- *Tissue-resident macrophages:* during embryogenesis (yolk sack and liver) Mfs colonize specific areas of the GIT. After birth, facing the external environment, these areas are occupied by monocyte-derived Mfs (that became the predominant population) until adulthood where we find both embryonic and monocyte-derived Mfs co-existing but not co-localizing.
- *Mature macrophages*: can often refer to the last step of maturation (the "waterfall") i.e. Mf3 or tissue resident Mf4. They exhibit features of less reactive and loss of plasticity Mfs (e.g. low or absent production of pro-inflammatory cytokines due to their adaptation of living in an antigen rich microenvironment such as that of the GIT).
- *Self-maintaining/self-renewing macrophages:* tissue-resident macrophages are long-lived; they self-renew and are not replenished by new blood monocytes. They are needed for tissue development and remodeling and able to survive *in situ*.

1.3.3 NEUTROPHILS

Neutrophils are the most abundant leukocytes and immune cells in circulation. They belong, together with basophils and eosinophils to the polymorphonuclear leukocytes or **granulocyte** categorization given their morphological features of nuclei shapes and cytoplasmatic granules respectively. Granulocytes together with mast cells derive from the common myeloid progenitor cells (Figure 3). Neutrophil production in the bone marrow can increase rapidly and hence their percentage in the blood increases in response to infections. They are not tissue-resident cells but are quickly and abundantly recruited from circulation into the tissue during active inflammation and tissue damage in CD[144,145,146] even in absence of infections

and correlate with disease severity[147]. Hence, as the disease progresses neutrophils are continuously recruited to the tissue (Figure 4). They are short-lived cells with a circulating half-life of 6–8 h[148], and mechanisms such as release of cytokines and growth factors by other cells (e.g. GM-CSF, IL-8, TNF-α) as well as microbial particles such as lipopolysaccharide (LPS) contribute to sustained recruitment of neutrophils in the tissue.[149,150] Neutrophils can perform the following functions: e.g phagocytosis, generation of neutrophil extracellular traps (called also NETosis), degranulation and generation of reactive oxygen species. Macrophages and neutrophils are the cells with the greatest ability to perform phagocytosis and have most often overlapping phagocytosis-related mechanisms such as types of intracellular receptors employed, and as such described side by side in basic immunology books.[151] All the abovementioned neutrophil functions facilitate killing of bacteria and clearance of the tissue that can also be combined rather than acting alone.[152] For instance, capturing of bacteria by NETs makes it more efficient for neutrophils to phagocytose these trapped bacteria. Once they accomplish their function they die e.g. by apoptosis. **Apoptotic neutrophils can be eliminated by efferocytotic Mfs** residing in the tissue and hence tissue clearance after inflammation is re-established. In CD, neutrophils' regulatory functions, which normally serve to maintain homeostasis in health, instead contribute to CD disease by impediment of healing, barrier damage and disease symptoms worsening.[153] For example, neutrophil extracellular traps exacerbate tissue damage during active CD and compromise the intestinal barrier integrity during experimental colitis (Figure 4).[154,155]

1.4 INTESTINAL FIBROBLASTS IN HEALTH AND CD

Fibroblasts are mesenchymal cells that contribute to the organs´ stroma (connective tissue) by producing various proteins/components (e.g. collagen) needed for structural holding and homeostasis of the tissues. Compared to all other organs, human GIT fibroblasts are transcriptionally very different, for instance in genes related to **extracellular matrix** (ECM) remodeling and signaling ligands, suggesting a specific functional diversity for the GIT.[156] Following intestinal damage, tissue-resident fibroblasts can become myofibroblasts, a type of specialized contractile fibroblast expressing alfa-smooth muscle actin (α-SMA).[157] Fibroblasts are cells conventionally

associated with fibrosis and tissue repair and not so much with renewal or inflammation. Nonetheless, their activity can influence inflammatory processes, other cells adhesion, migration, and proliferation ability in the tissue.[158] In addition, fibroblasts are considered **sentinel immune-like cells** because of their overlapping functions with innate immune cells such as the presence of certain PPRs and the ability to produce inflammatory factors in response to microbes.[159,160] The exact processes of how intestinal fibroblasts are able to initiate healing but promote inflammation remain vague.[157] However, one recent theory is that during fibro-stenotic CD there will be location- and disease-specific fibroblast subsets, normally not established/activated in non-affected intestinal areas, that are influenced for instance by local inflammatory monocytes.[161] Given the ongoing intestinal inflammation in CD, cytokines, such as TGF-β1 abundantly produced by Mfs, activate intestinal myofibroblasts, contributing to the intestinal fibrotic tissue formation.[162] Consequently, both TGF-β1 and collagen activity corresponds to the inflammatory infiltrate (transmural) in CD.[163] Regarding fibroblast subtypes, the literature has not reached a consensus of the surface markers to use in defining functionally active fibroblasts. However, regarding CD lesions some markers appear more frequently in literature. For instance, fibroblasts closest to the fibrotic areas in CD express high levels of fibroblast activating protein (FAP)[164,161] and attempts made to inhibit FAP have been successful in showing a decrease in ECM protein expression (collagen type I)[165] and could potentially be considered for further treatment investigations.

1.5 INFLAMMASOMES AND TREM-1 RECEPTOR

Receptors on innate immune cells (such as Mfs) are key components of innate immune responses, which are non-specific responses to e.g. danger signals, foreign substances, or MAMPs, and are used to facilitate the first line of defense. Hence, a number of innate receptors are primarily responsible for establishing the host–microbial communication in the intestinal mucosa.[166] In this chapter, I write about the main receptors investigated in this thesis. These are PRRs and PRR-like receptors, focused on inflammasomes, signaling receptor TREM-1, and the downstream effectors involved in the signaling of these. Such receptors locate either on cell surfaces, in the cytoplasm, or in endosomes and are involved innately in the recognition of microorganisms,

parts of microorganisms or byproducts of cell metabolism that serve as initiating signals.

Table 2: Types of pattern recognition receptors.[167]

PRRs	Full Name	Cellular location
NLRPs	Nucleotide oligomerization domain (NOD)-like receptors or Nucleotide-binding oligomerization-Like Receptor PYD domain	Cytoplasm
TLRs	Toll-like receptors	Membrane, Endosome
CLRs	C-type lectin receptors	Membrane
ALRs	Absent in melanoma-2 (AIM2)-like receptors	Cytoplasm
RLRs	Retinoic acid-inducible gene-I (RIG-I)-like receptors	Cytoplasm
PRR-like		
TRIMs	Tripartite motif proteins	Cytoplasm

1.5.1 INFLAMMASOME COMPLEXES

Inflammasomes are cytosolic protein complexes/platforms assembled in response to various signals deriving from the extracellular or intracellular environment (e.g. microbes, metabolism products). The inflammasome was discovered in 2002 as considered an important molecular platform and pathway of innate immunity by which the activation of inflammatory caspases is triggered with processing of inactive cytokine pro-IL-1β into IL-1β as the final consequence.[168] Since the first discovery of the inflammasome in the macrophage cell line THP-1, inflammasomes have been widely studied in this cell line and gradually expanded to other cells. Whereas an overwhelming amount of data in the past 21 years derives from THP-1 cells,[169,170,171,172] it is now known that inflammasome activity is cell-, tissue-, species-, and disease-specific.[173] Regarding the intestine, in recent years a number of cell types have been shown to utilize inflammasomes during infections and diseases making inflammasomes innate key immune platforms for e.g. IECs, Mfs, monocytes and neutrophils.[173,174,175,176]

1.5.1.1 STRUCTURE, ASSEMBLY AND ACTIVATION

Typically, an inflammasome is constituted by a sensor, an adaptor and effector molecules. Inflammasome sensors give the name to the inflammasome complex. For instance, an inflammasome employing NLRP3 will be called the NLRP3 inflammasome complex or platform. Several inflammasome complexes exist and their sensors belong to different receptor classes, and can be combined with different effectors depending on the inflammasome type.[177] One broad classification of inflammasome signaling is that of canonical or non-canonical inflammasomes defined by their use of Caspase 1 (Casp1) or Casp4/5.[178,173] In the canonical inflammasome, the sensor is a cytosolic PRR, the expression of which increases in the presence of a signaling ligand. For instance, the most known activation pathway of NRLP3 occurs once bacterial components (e.g. LPS) binds to TLRs (Figure 7, step 1). This is referred to as "first signal" and triggers translation of increased expression of NLRP3 in the cytosol (Figure 7, step 2). The NLRP3 sensor then needs a "second signal" to assemble with the other components to form the complex.[169] To this end, ATP or Nigericin (Figure 7, step 3) induce the assembly of the NLRP3 inflammasome complex. This last is an assembly where the NLRP3 sensor binds to the adaptor protein ASC by using a shared PYD domain and this then triggers the binding of ASC with pro-casp1 via the shared CARD domain (Figure 7, step 4). Pro-casp1 is then activated to a proteolytically active enzyme called Casp1 that upon its release induces the cleavage of pro-ILβ and pro-IL-18 into active forms IL-1β and IL-18[179] (Figure 7, step 5). These active cytokines then get released from the cells influencing context specific pro-inflammatory processes (Figure 7, step 6). The fate of the cell, in Figure 7 shown as activated Mfs, is either a form of inflammatory cell death called pyroptosis and release of pore-forming gasdermin D[180] or the cell gets hyperactivated.

Table 3. Type of the PRR that each inflammasome sensor belongs to.

Sensor / Inflammasome PRRs	**NLRP1**	**NLRP3**	**NLRP6**	**MEFV**	**AIM2**	**NLRC4**
NLRPs	✓	✓	✓			
NLRC						✓
TRIM				✓		
ARLs					✓	

Nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs) containing the caspase activation and recruitment domain (CARD; NLRC), and the pyrin domain

(PYD; NLRP). TRIM: tripartite motif proteins; MEFV (or TRIM20): mediterranean fever; ARLs: absent in melanoma 2 (AIM2)-like receptors (ALRs); GSDMD: gasdermin D.

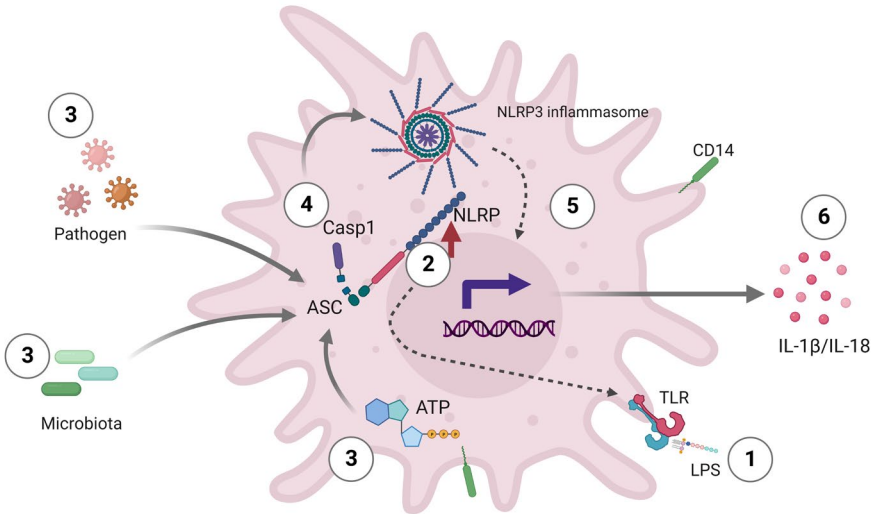


Figure 7. Core events triggering inflammasome activation up to structural changes and assembly of the complex. Shown as an example is the NLRP3 inflammasome activated macrophages. This representation regards the most known pathway with the shown signals also called canonical inflammasomes. The signaling events are presented regardless of tissue or disease just as an example. Abbreviations: NLRP, Nucleotide-binding oligomerization-Like Receptor PYD domain (NOD-like receptor); Casp, caspase; ATP, Adenosine triphosphate; TLR, toll-like receptor; LPS, lipopolysaccharide; CD, cluster of differentiation; IL, interleukin; ASC, apoptosis-associated speck-like protein containing a caspase activating and recruitment domain.

1.5.1.2 FUNCTIONS IN INNATE IMMUNITY AND CD

Inflammasome complexes are expressed by multiple immune and non-immune cells in the intestine and contribute to the host defense against infections.[173] For instance, through the NLRC4 inflammasome complex on $CD11b^{+}F4/80^{+}$ phagocytic Mfs, and by NLRC4 deficient mice, it was shown to promote host defense to pathogenic *Salmonella* via recruitment of neutrophils to fight the infection.[181] Beside pathogens, microbiota are equally able to induce inflammation, in response to damage. For instance, via the NLRP3 inflammasome activation *Enterobacteriaceae* induces robust IL-1β release analogous to pathogen *Salmonella*.[182] On the other hand, incorrect activation of the NLRP3 inflammasome complex can translate into damage of the intestinal mucosa.[173,183] This was shown by for instance administering

medications such as colchicine, known to act on blocking NLRP3 inflammasome activation, that have been shown to prevent other drug-induced intestinal damage.[183] Another important inflammasome, NLRP6, was studied in relationship to the activity of newly recruited inflammatory monocytes in DSS-induced colitis[184] and was involved in the declined progression of colitis in IBD mice models.[185]

The *MEFV* (Mediterranean fever) gene, and its corresponding protein pyrin, is a special case of inflammasome sensor because in contrast to most inflammasome sensors which are NLR-derived, MEFV belongs to the tripartite motif-containing (TRIM) superfamily of cytosolic receptors. MEFV is considered a candidate PRR but is also an inflammasome since it can bind to ASC to form a casp1-inflammasome-complex and pyrin levels directly relate to IL-1β processing in monocytes and macrophages.[186] In response to IFNs, during innate immune responses, there is an increased expression of TRIMs,[187,188] making these receptors participants of innate immune responses similar to other PPRs. From the start, MEFV was identified as the main gene responsible for the Familial Mediterranean fever, which is an genetic autoinflammatory disorder, but it is also involved in a diverse range of biological processes that are associated with innate immunity, genetic disorders and cancers.[187] Regarding IBD, mutations in MEFV are also frequently detected in a subgroup of IBD patients and are associated with extra-intestinal manifestations.[189] The MEFV inflammasome, as an innate immune sensor, is known to sense bacterial toxins. These bacteria toxins make use of the MEFV inflammasome complex, indirectly inactivate guanosine triphosphatase RhoA (GTPase-RhoA) and finally the process results in IL-1β release.[190,191] Several intermediate proteins are involved in this signaling, such as serine-threonine kinases PKN1 and PKN2[190] which were also quantified in paper I. The process of GTPase-RhoA inactivation is known also as a phagocytosis suppression mechanism induced by pathogenic bacteria, including *Clostridium difficile* in the intestine.[189,190,191] It is not clear if there is a direct connection between the MEFV inflammasome complex and phagocytosis. However, upon polarisation of monocytes into monocyte-derived Mfs which are better at phagocytosis, pyrin levels and associated IL-1β secretion were shown to decrease.[186]

1.5.2 TREM-1 SIGNALING RECEPTOR

Beyond classical PRRs, innate immune cells express other signaling receptors that sense the local intestinal microenvironment and influence immune

response in patients with CD. One of these receptors is Triggering Receptor Expressed on Myeloid cells 1 (TREM-1). TREM-1 is an immunoglobin superfamily member receptor characterized by an extracellular Ig-like domain, transmembrane region, and a cytoplasmic domain without motifs for signaling.[192] Having no end signaling motifs, the receptor requires a second receptor called immunoreceptor adaptor protein DNAX activation protein 12 which provides a negatively charged aspartate that interacts with a positively charged lysine on TREM-1.[192,193] Regarding the role of TREM-1 in innate immunity, TREM-1 is expressed in a number of innate immune cells including monocytes, neutrophils and Mfs and in multiple organs counting the intestine.[194,195,146,196] TREM-1 up-regulation and responses can be triggered by MAMPs such as LPS[195] and peptidoglycan[197] and cytokines like TNF. Upon activation, the TREM-1 pathway is a **powerful amplifier of inflammatory responses**.[198,199] Particularly, TREM-1 can synergize with other PRRs, especially TLRs, to amplify an ongoing pro-inflammatory cycle.[200] Ligands of TREM-1 seem to be tissue- and disease-specific, but since they are common ligands recognized by a number of other PRRs, it has been challenging to establish a full and precise spectrum of "*what is who and when"*.[196] Yet, it is believed that despite the unclarity of the ligand type, therapeutic targeting of the TREM-1 receptor for inflammatory diseases is promising and will potentially not increase infections as $TREM1^{-/-}$ mice were still able to maintain control of pathogens.[201] However in the context of CD, this has yet to be established for the commensals, given that there is a leakage of the lumen content into the lamina propria where $TREM\text{-}1^{+}$ immune cells reside.

It is not clear if TREM-1 is completely absent or slightly expressed during homeostasis. *TREM-1* gene expression and TREM-1 expressing Mfs were shown to be nearly absent in the LP of non-inflamed control colonic tissue.[199] However, in a different study, TREM-1 was shown to be present in healthy colonic tissue with lower expression by $CD206^{+}CD68^{+}CD163^{+}$ mature Mfs as compared to monocytes.[108] In contrast, $CD206^{-}$ healthy colonic Mfs, which have a more immature phenotype and are more similar to monocytes, seem to express an intermediate level of TREM-1.[108] Likely, the low or absent presence in homeostasis is to avoid vast inflammatory reactions and intestinal harm given that TREM-1 is a potent amplifier of inflammation. This is not the case **during CD.** $TREM\text{-}1^{+}$ Mfs appear to increase during CD, and they have been defined for instance as $TREM1^{+}CD68^{+}$ Mfs and $TREM1^{+}CD14^{+}CD89^{+}$ Mfs present in increased frequencies in the colonic mucosa of patients with CD.[199] Furthermore, concerning a potential role in disease pathogenesis, a number of important findings have emerged that have placed TREM-1 under the spotlight

both for CD and UC. First, TREM-1 expression is increased in active CD areas[199], and inhibition[202] or knockout[201] of the TREM-1 receptor attenuates the severity of colitis. Second, TREM-1 was proposed as a biomarker for non-response to anti-TNF therapy.[203] As the response rates of patients treated with anti-TNF agents is only approximately 30%, there is a huge and equal need for predictive biomarkers as well as new treatments.[204,25] However, there is still confusion on the role of the TREM-1 receptor *per se* given that TREM-1 exists as membrane bound as well as soluble, and contradictory results for transcriptional alterations have been reported regarding anti-TNF response related to the two forms.[204]

1.5.3 *POINT OF VIEW*: TARGETING PRRS AND PRR-LIKE SIGNALING RECEPTORS IN CD

If one thinks about the GIT microenvironment, it makes sense that intestinal cells have inflammasomes and TREM-1 and that they will have an important function there. This is because the intestine has a mucosa with a huge surface which encounters loads of bacteria, antigens, and food components. Residing in such microenvironment, intestinal myeloid cells have PPRs or PRR-like receptors to recognize danged signals and foreign substances, communicate this to other cells and help the intestine to resolve invasion from microbes and potential inflammation. During health, inflammasomes in the intestine recognize bacterial and cell metabolism products contributing to the physiological cell machinery. During intestinal infections and disease inflammasome function changes, they conceivably increase in activity and the ligands they recognize are possibly disease-related. The TREM-1 signaling pathway seems to be functionally present, but low expressed, in myeloid cells during homeostasis suggesting that the pathway is needed for the physiological functioning of the human GIT. During CD, with the increase of a myeloid cell recruitment from blood to the tissue, and changes in tissue content in myeloid cells due to the disease, these receptors increase in activity and the myeloid cell subsets seem to shift subsets and consequently function. Potential drug-targeting of NRLs, MEFV and TREM-1 should consider specific patient subgroups and predict/prevent the consequences of inhibiting a receptor that is needed for a protective physiological function (e.g. protection against infections).

2 AIMS

The intent with this chapter is to provide a summary of the aims stated in each of the papers. Aims and hypothesis go often hand by hand, hence it would be (almost) impossible not to think about the grounds the research ideas generated from.

The overall aim of this thesis was to determine innate immune cellular and molecular mechanisms driving intestinal inflammation and fibrosis in patients with active CD. Hypothesis driven specific main aims were to:

- Determine inflammasome complexes components expression levels, and their downstream effectors/cytokines, in patients with CD compared to controls and UC.

- Establish the relationship of inflammasome complexes to disease activity and systemic inflammation.

- Identify and classify Mf subsets in the intestine of patients with ileal active CD compared to non-inflamed subjects (controls).

- Determine innate immune TREM-1 receptor and its downstream effectors/cytokines associated, expression on Mfs subtypes and correlation with CD disease activity.

- Establish the involvement of FS from CD patients, representing the intestinal microenvironment, on M2MQ and intestinal fibroblasts in relationship to inflammatory and fibrotic processes.

- Compare patient-subgroups, iCD and sCD, and their FS capacity in polarizing M2MQ and intestinal fibroblasts in relationship to inflammatory and fibrotic processes.

3 METHODS AND EXPERIMENTAL PROCEDURES

In these chapter I write about relevant methodologies used in my thesis work. The chapter is meant to describe the rationale and critically discuss the usage, and comparison between methods and literature. More details, which are not repeated here, and other assays can be found in the respective *Methods* section of each specific paper found in the printed thesis book.

3.1 HUMAN SAMPLES

All the findings of this thesis are obtained from human samples and primary cells. Human samples obtained under the respective ethical permits used in this thesis included:

- Fecal samples from patients with CD and healthy subjects
- Mucosal tissue (ileum, colon) from patients with CD undergoing surgery.
- Mucosal tissue from patients with UC (colon, rectum).
- Serum from patients with CD and healthy gender- and age-matched subjects.
- Non-inflamed, non-affected control surgical tissue (ileum, colon) from colorectal cancer patients undergoing surgery (controls).
- Healthy mucosal biopsies from study subjects undergoing routine checks for non-inflammatory conditions.

Table 4. Tissue and fecal samples

Cohort	Paper I	Paper II	Paper III
CD	x	x	x
UC	x	x	
Control	x	x	
Healthy	x		x

The following primary cells were isolated/used:

- Monocytes, neutrophils and bulk PBMCs from blood of healthy subjects.

- Bulk immune cells and IECs from ileum of patients with CD and non-inflamed subjects (controls).
- Primary intestinal fibroblast cell line.

Table 5. Type of samples used in each study.

Type of samples	Paper I	Paper II	Paper III
Fecal supernatants			x
Mucosal tissue	x	x	
Conditioned media	x	x	x
Monocytes		x	x
Neutrophils			x
PBMCs	x		
Serum	x		
Fibroblasts			x

Of note, although a lot of the research on the topic is performed in animal models and cell lines, human studies are increasingly needed. Animals and cell lines provide applicable tools for functional studies, such as cells fate-mapping or gene knockout otherwise not possible to conduct in humans, however they do not fully reflect the real biological circumstances of the human immunobiology. The main advantage of using human material is its closeness to veracity and directly translatable findings for patients. Nevertheless, the main disadvantage is that humans come with a broader natural variability, subgroups, and disease-related parameters (location, complications, disease activity) and hence require larger sample sizes that are not always easily obtainable.

3.1.1 IBD SAMPLES

During this thesis work, I have used samples from adult patients suffering from CD and UC residing in Sweden and Denmark. Regarding the Swedish cohort (**Paper I and II**) used for mucosal tissue, in addition to chronic inflammation, with periods of relapse and remission, these patients were at a stage of disease, independently of the disease duration, where they had developed various complications (fibrosis, stenosis, strictures, fistulas, dysplasia) that drives some patients to undergo surgery. Hence, tissue samples were collected after the surgical removal of the tissue and blood samples were taken just before the

patient entered the surgery at Sahlgrenska Hospital in Gothenburg, Sweden. From these IBD patients, demographics and clinical parameters such as disease activity index/score were also available. Regarding the Danish cohort, that was used for fecal samples (**Paper III**), patients with CD and healthy subjects were from the baseline of an intervention trial.[205,206] These patients were classified into subgroups: patients with inflammation only (named iCD) or inflammation and stenosis (named sCD).

3.1.2 CONTROL AND HEALTHY SAMPLES

Regarding controls and healthy subject samples, human tissue and blood was obtained conditional to the type of analysis. In general, most of the control mucosal tissue was from non-affected/non-inflamed tissue of colorectal cancer patients undergoing surgery and a few healthy tissues from biopsies of persons undergoing endoscopy for health screening. Healthy tissue from humans is always an obstacle when it comes to wanting to obtain tissue samples from "entirely" healthy individuals. Despite this being very relevant, the GIT sampling for purely research purposes is perceived as invasive for the study subjects and time consuming for the health care system. In addition, complications (such as hemorrhages, perforations and infections) due to biopsy sampling in the terminal ileum is considered debatable given the anatomy of this area.[207] Hence, alternative controls, that do not require healthy individuals to undergo a biopsy only for research, are of choice. In our specific case the amount of tissue we needed to perform multiple experiments (Paper I and II) was considerable hence the available approach to get this amount of tissue was from non-inflamed/non-affected tissue of colorectal cancer patients removed during surgery. Surgeries in these patients almost always involve the removal of the affected tissue surrounding the tumor area (for preventive reasons), some of which can be used for research in addition to pathological investigations. However, for some analyses where we only needed a small biopsy, some samples were from biopsies of persons undergoing endoscopy for health screening.

Regarding blood samples, they were used for serum analyses or purification of monocytes, neutrophils or PBMCs. Samples were from blood or buffy coats of healthy blood donors. Blood samples are easy to access, hence people have no difficulty in donating blood for research. For the serum samples, I decided to get sex- and age-matched subjects which allowed to avoid the detection of inflammatory proteins changes due to age differences (Figure 8).[208] Given the

slow advancement from bench to bedside of IBD research, more and more studies have come to shed light regarding the demographic parameters as influential to the treatment. For instance, immune cell types in UC associated with anti-TNF response have a specific sex-dependent profile.[209] Similarly in the case of fecal samples, it is easy to obtain samples from healthy subjects hence paper III included a cohort collected at the same time as the patients with CD.

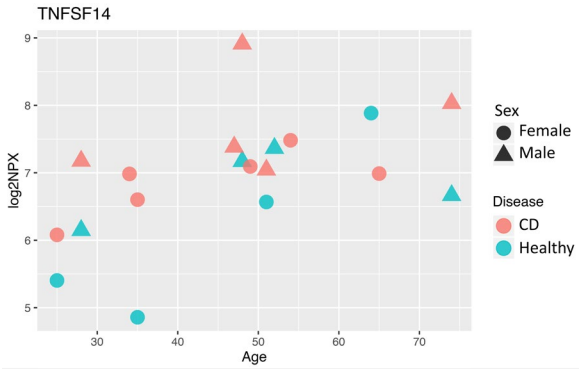


Figure 8: Importance of sex- and age-matched serum collection. Example of a serum protein (TNFSF14) that correlated with age, in age matched samples, regardless of CD or healthy subjects. The correlation value is R=0.526 and the significance value p=0.021. The graph was generated with published data from Gorreja et al 2022.[1] TNFSF14, Tumor necrosis factor superfamily member 14; NPX, normalized protein expression measured with PEA assay.

3.2 BLOOD AND PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMCS)

Human peripheral blood mononuclear cells (PBMCs) were analyzed in all three papers of this thesis for different purposes. PBMCs were isolated from either whole blood or buffy coats using density gradient centrifugation.[210] First, PBMCs were used to analyze gene expression of both healthy and patients with CD (Paper I) to investigate inflammasome expression of key inflammasome component genes. Second, PBMCs were used for purification of the $CD14^+$ monocyte subset which were freshly seeded and stimulated (as described below) to mimic the arrival of monocytes in the intestine (Paper II). Third, PBMCs from healthy buffy coats, which contain a concentrated number of cells, were used for purification of $CD14^+$ monocytes to be polarized into M2MQ (Paper III). Finally, whole blood was used for purification of granulocytes (Paper III).

3.3 INTESTINAL ILEAL TISSUE PROCESSING AND PRIMARY CELL ISOLATION

Primary cells from human intestinal mucosa (ileal, ileocolonic) of both non-inflamed study subjects (control tissue) and patients with CD were isolated by a well-known and widely used approach employing chemical, enzymatic and mechanical means.[75,211] The approach is described in details in each published paper (Paper I and II) and schematically below for simplification (Figure 9). Briefly, the intestinal tissue is processed fresh to allow for the viability of most cells. Isolation of IECs, from the top layer, are obtained by the help of the chelating agent ethylenediaminetetraacetic acid (EDTA) and incubation with continuous stirring to facilitate the dissociation of the tissue. EDTA chelates/binds to calcium and stops cell aggregation and promotes detaching of adherent cells (e.g. inhibit adhesion molecules catherins), and helps detach the IECs/epithelial layer from LP layer of the mucosa.[212] Isolation of IECs with EDTA is a widely used procedure for the intestinal tissue, although other agents are used as well such as ditiotreitol (DTT) and are equally good in performing the same action while preserving the cells viability.[213] At the end of this step an IEC suspension is obtained, referred to in the papers as *enriched epithelial cells*, that was used to investigate the gene expression of inflammasome components and TREM-1 receptor as well as flow cytometry analysis.

Next, for the processing of the LP layer, enzymatic and mechanical digestion methods were combined. Enzymatic digestion included the use of Liberase DL (Dispase Low) which allows dissociation of the tissue and release of immune cells in a suspension. This was combined with both incubation at 37°and continuous stirring to help dissociation but also because Mfs are highly adherent cells that could easily attach to plastic surfaces. At the end, mechanical breakage is shortly applied to make sure that no connective tissue withholds the cells. Liberase digestion (synonym collagenase) is a widely used enzymatic method for intestinal tissue processing and isolation of MNPs[214] and immune cells in general. From this Liberase and mechanical digestion a bulk LP cell suspension was obtained, called *enriched LP suspension* in the papers. The LP cell suspension was used for flow cytometry analysis, gene expression analysis and cell culture as described below in the respective sessions. Lastly, it has to be pointed out that with the developing use of the single cell technology, new studies are emerging where effect of dissociation protocols (enzyme types, times of digestion) are compared on how these affects immune

cell composition.[215] To date however, there is no current agreement in literature on how different dissociation protocols affect MNPs specifically. However, this would be of importance to study not only to assess that there is a good viability but also regarding various subsets and surface markers being affected. This is especially important when it comes to Mfs given that the absolute numbers of Mfs in the intestine are very low and even small changes, in the context of the entire immune cell pool, could be of major relevance for Mfs.

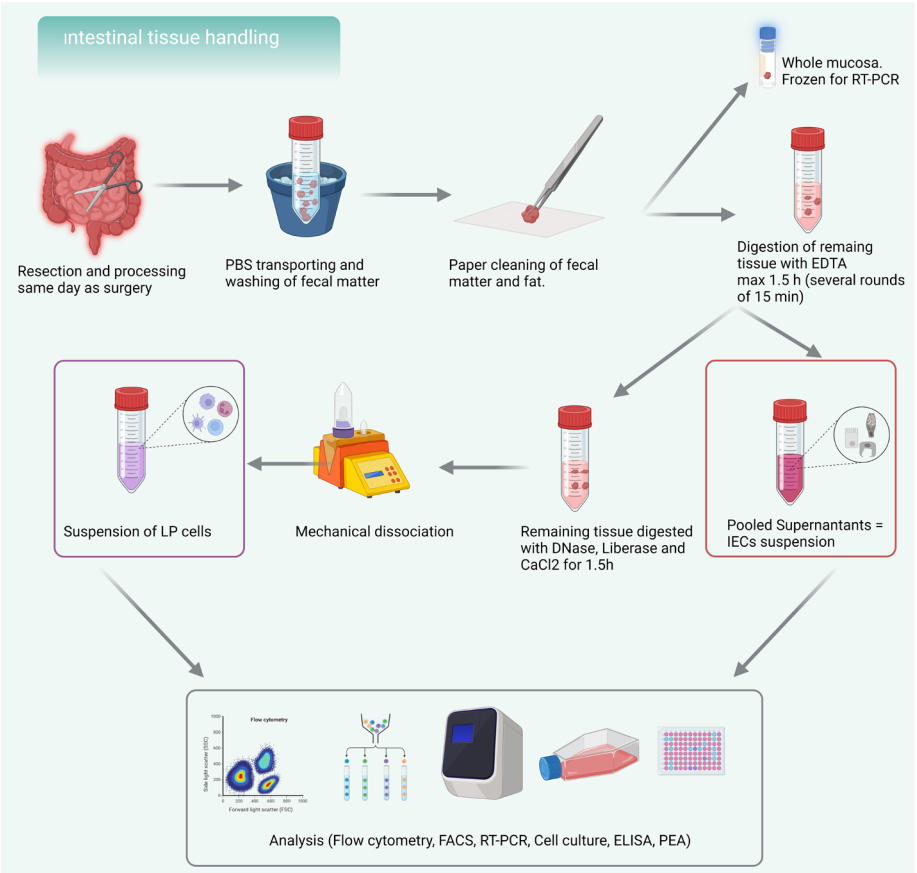


Figure 9: Step-by-step clarification of intestinal tissue handling, processing, and digestion. Details are found in the respective Paper I[1] and II[2]. PBS, Phosphate-buffered saline; RT-PCR, Reverse transcription- Polymerase chain reaction; EDTA, ethylenediaminetetraacetic acid; LP, Lamina propria; CaCl2, Calcium chloride; IECs, Intestinal epithelial cells; FACS, Fluorescent activated cell sorting; ELISA, enzyme-linked immunosorbent assay; PEA, Proximity extension assay.

3.4 CELL CULTURES, STIMULATIONS AND FUNCTIONAL ASSAYS

Different primary cell or cell line cultures were performed in this thesis, depending on the aim of each paper. Below is shown a table summary and subchapters thereafter describe the use of each cell type.

Table 6. Cells culture list showing derivation and cultured media used.

Cells (Full name)	**Species, Type, Derivation**	**Culture Media**
LP cells (Lamina propria cells)	Human, Primary, Mucosa	Roswell Park Memorial Institute (RPMI) 1640 Medium, L-alanyl-L-glutamine, HEPES, FBS, gentamicin
$CD14^+$ Monocytes	Human, Primary, PBMCs	Roswell Park Memorial Institute (RPMI) 1640 Medium, L-alanyl-L-glutamine, HEPES, FBS, gentamicin
M2MQ (M2 macrophages)	Human, Primary, PBMCs	Serum-Free Base Media, M-CSF. Iscove´s Modifies Dulbecco´s Medium, FBS, L-glutamine, gentamicin
CCD-18Co (Intestinal fibroblasts)	Human, Primary, Cell line	Eagle´s Minimum Essential Medium (EMEM), FBS, gentamicin
$CD66b^+$ Neutrophils	Human, Primary, Blood	Roswell Park Memorial Institute (RPMI) Medium, FBS

Concentrations and respective commercial brands are described in each paper. HEPES, 2-[4-(2-Hydroxyethyl) piperazin-1-yl] ethane-1-sulfonic acid; FBS, fetal bovine serum.

3.4.1 LP CELL CULTURE

Cell suspension of bulk LP tissue layer (as obtained on Figure 9) were cultured for 1 day to obtain conditioned media/supernatants representing the LP microenvironment from inflamed CD and controls. To make sure the supernatants were cell/particle/bacteria/debris free, supernatants were filtered. First, these LP supernatants were used for the quantification of IL-1β (paper I), TNFα (paper II) or 92 inflammation-related proteins (paper II) with proximity extension assay (PEA). **IL-1β** was measured as the main end-point effector protein of the inflammasome activation cascade in the LP propria. IL-

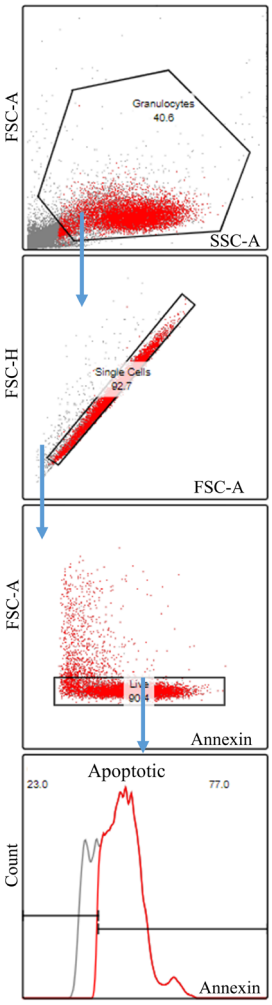
1β protein measurement was important given that the other inflammasome-related measurements in the tissue were all at the gene expression level. IL-1β is a pro-inflammatory cytokine released predominantly by immature Mfs.[216] **TNFα** was also measured as an important pro-inflammatory cytokine released from immature Mfs in the LP.[217] The 92-protein panel was an exploratory analysis to have an overview of the inflammatory profile of the LP supernatants, which were then used to stimulate $CD14^+$ monocytes to mimic their arrival in the intestine as newly-recruited monocytes. The aim was to obtain monocytes in an intestinal like macroenvironment that would resemble their transformation from monocytes into immature macrophages.

3.4.2 $CD14^+$ MONOCYTE CELL CULTURE

$CD14^+$ monocytes were cultured because they highly express TREM-1 receptor[218] and are the precursors of newly-recruited Mfs in the intestine. Peripheral $CD14^+$ monocytes were isolated from PBMCs of buffy coats from healthy subjects and were cultured for 5 h with different conditions (Paper II): with or without peptidoglycan (from *Staphylococcus aureus*) which represents one of the known ligands for TREM-1 and used here as positive control; TREM-1 agonist, TREM-1 antagonist, anti-TNF or isotype control antibodies (IgG1, IgG4). Furthermore, to mimic the transformation of **$CD14^+$ monocytes when arriving in the intestine**, some monocytes were cultured with conditioned media of LP cells either from inflamed CD or controls. Finally, stimulated $CD14^+$ monocytes underwent gene expression analysis with either RT-PCR or mRNA counts (subchapter 3.6) using a Myeloid Innate immunity gene set[219].

3.4.3 NEUTROPHIL CELL CULTURE AND APOPTOSIS INDUCTION

During inflammation, neutrophils recruited in the intestine can undergo **apoptosis** and produce signals that can attract Mfs to eliminate these apoptotic cells, a function performed by Mfs named **efferocytosis**.[220] Efferocytosis is very important for clearance of the tissue hence resolution of inflammation.



Sample	Annexin+ cells %
Time 0	9 %
Time24 FBS 0%	68 %
Time24 FBS 1%	77 %
Time24 FBS 5%	76 %
Time24 FBS 10%	68 %

Peripheral blood **CD66b$^+$ granulocytes** were used in Paper III and were purified from whole human healthy blood. CD66b is a glycoprotein associated with the aggregate formation of human neutrophils[221] and the majority of CD66b$^+$ in the blood are neutrophils that represent the majority of granulocytes in the blood. Neutrophils are short-lived and very sensitive cells hence experimental use after isolation was performed immediately. Furthermore, the aim was to culture the cells to bring them to an apoptotic state which *per se* would imply losing a good number of cells. In short, CD66b$^+$ granulocytes were purified from fresh whole blood and residual red blood cells were lysed, a crucial step given that the whole blood isolation leaves much more red blood cells behind compared to i.e. isolation from PBMCs. Isolated cells were stained with a tracer (culture dye), and *spontaneous apoptosis* was induced by incubating the cells in RPMI containing **1% FBS**. To eliminate NETs generated, the cells were treated with a gentle dissociating solution and filtered. Apoptotic status was confirmed with flow cytometry by using **AnnexinV.** Several factors made this in-house assay time-consuming and challenging to set up, given that a myriad of methods in literature exist, all claiming to induce apoptosis on various cells including neutrophils. Hence, a lot of testing was required to establish what worked for our primary cells and is worth bringing up some discussion points.

Figure 10. Back gating for identifying Annexin$^+$ (apoptotic) granulocytes. Table shows the % of apoptotic cells by using various concentrations of FBS. FSC-H, forward scatter- hight; FSC-A, forward scatter-area: SSC-A, side scatter-area.

First, some studies use the method of *spontaneous apoptosis induction* by just culturing the cells in culture media and FBS[222] (similarly to the one I performed in paper III). Other studies use *ultra violet radiation-induced apoptosis*[223] or *chemically-induced apoptosis* that involves the use of a compound that sends the cells to apoptosis, for instance with potassium chloride solution[224] or staurosporine[225,226]. The difference between

the first and the other two methods is that the first method is more physiological but requires a longer time (24h incubation) while the other methods require less time to induce apoptosis. However, for the second method it is not clear if the chemicals or radiation used have a secondary effect on the cells. Other studies even use a mixture of the two abovementioned methods by combining both incubation and chemically- [227] or radiation-induced apoptosis[228]. Yet, the literature considers all the three abovementioned methods biologically relevant in inducing cell apoptosis.

Second, regarding the *spontaneous apoptosis induction* performed by incubating neutrophils with cell culture media for 20-24 h, studies do not seem to agree on the amount of FBS to use in the culture ranging from 10%[229] to 1% FBS[222]. For this purpose, I tested concentrations of FBS able to give the highest number of apoptotic neutrophils (Figure 10) and the use of FBS 1% gave a higher apoptotic rate and was used in paper III.

3.4.4 M2MQ CELL CULTURE AND EFFEROCYTOSIS ASSAY

Anti-inflammatory or tissue-resident Mfs are present in very small numbers in the human intestine both in health and disease.[65] We needed a model to represent these Mfs while having sufficient number of cells for functional experiments (paper III). Hence, the *in vitro* equivalent of tissue resident Mfs M2MQ were used. M2MQ were obtained by culturing purified $CD14^+$ monocytes for 6 days with M-CSF and 1 day with LPS. Macrophage colony-stimulating factor (M-CSF) is a hematopoietic growth factor that controls the functional activation of monocytes by controlling their proliferation and differentiation.[230] On the other hand, exposing M2MQ to lipopolysaccharide (LPS), as a MAMP present on the $gram^-$ bacterial cell wall that can signal through CD14, induces further M2MQ activation. The establishment of this M2MQ reproducible cell culture was assessed by: (1) measurements of **$IL\text{-}10^{high}$** cytokine release in the supernatants, via ELISA, which is one of the main cytokines released from these cells upon activation; (2) confirmed to be $TNF\alpha^{low}$ and $IL\text{-}12p70^{low}$ in the supernatants, via ELISA, which are instead cytokines characterizing M1MQs; (3) microscopical

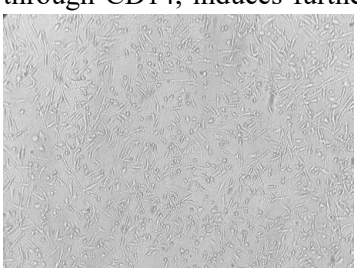


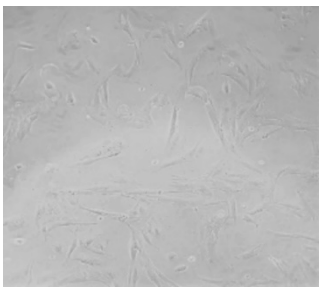
Figure 11. Microscopic view of activated M2MQs culture.

visualization and formation of Mfs filopodia "arms" (Figure 11) known to be lacking in monocytes; (4) confirmed to be $CD14^{+}CD163^{+}CD80^{-}$, via Flow cytometry, especially $CD80^{-}$ given that M1MQ are $CD80^{+}$ (Table 1).

M2MQ were used in paper III for stimulations with FS from patients with CD and healthy subjects and after stimulations the following were investigated: (1) secretion of anti-inflammatory and prototypical M2MQ cytokines/proteins in cell culture supernatants, (2) efferocytosis function to investigate if the FS-activated M2MQ could perform their natural-GIT ability of clearing up the tissue; (3) interactions with fibroblasts through the FS-conditioned media of M2MQ. These last three were thoroughly described in paper III.

3.4.5 FIBROBLASTS CCD-18CO CELL CULTURE

A primary cell line of intestinal fibroblasts, namely CCD-18Co, was used in paper III. CCD-18Co has been previously used in research related to CD.[161,231,232] For instance, most recently CCD-18Co fibroblasts were used to investigate their activation when exposed to inflammatory monocytes isolated from inflamed ileal tissue from patients with CD undertaking surgery for stenosis complication.[161] They are relatively easy to culture (Figure 12, left) and maintain while performing large experiments and a good static model to use when aiming to compare multiple treatments/stimulation (i.e. patients' material with healthy subjects). More precisely, in paper III CCD-18Co fibroblasts were used to investigate the effect of treatments with (1) with FS and (2) FS-conditioned media from M2MQ. FS were from patients with active CD presenting only inflammation (iCD), CD with stenosis and inflammation (sCD) and healthy subjects. FS-treated fibroblast supernatants were used to quantify the **inflammatory contribution** via IL-6, MCP-1 and IL-8 (treatment 1) and the contribution in **extracellular matrix formation** by measuring proteins collagen I and fibronectin (treatment 1 and 2). Furthermore, fibroblast surface (FAP, PD-L1, CD21) and intracellular proteins (α-SMA, TGFβ1), were determined via flow cytometry, to investigate



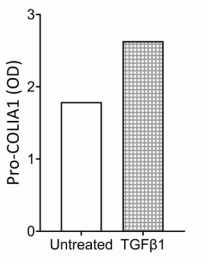


Figure 12. Intestinal CCD-18Co fibroblasts. Left: Morphology of cells. Right: Production of collagen I after 72h of stimulation with TGFβ1 5 ng/mL.

phenotypes affected by treatment 1 and 2. Finally, fibrosis-related gene expression was quantified on fibroblast cultured with treatment 2. To set up the cell culture time of stimulations and with respect to Mf experiments, fibroblasts were stimulated with TGFβ1 as positive control (Figure 11, right) which is a cytokine known to induce activation and collagen production[233] and in the intestine known to partially derive from tissue resident Mfs.[234]

3.5 FECAL SUPERNATANTS (FS)

Fecal supernatants (FS) refer to the small molecules remaining from the dilution and ultracentrifugation of fecal matter/samples (including the removal of whole bacteria content) as described in paper III methods and as previously reported.[139] Fecal samples used in this thesis were from patients with CD and healthy subjects. The CD subgroup was divided further into sCD and iCD as the aim was to investigate how CD differs from healthy but also if the FS from stenotic group (sCD) would have a different effect on fibroblasts and Mfs compared to the group with inflammation only (iCD) and healthy. Although FS contains several unidentified small molecules, the most measured are the metabolites. **Metabolomics**, in the case of fecal samples, is the investigation of end products of metabolism of microbiota present throughout the GIT. Hence, the metabolome of FS from the three groups was quantified with gas chromatography coupled to a tandem mass spectrometer as previously reported.[235,236] Metabolomic profiling of samples obtained from patients with CD has been previously performed to identify disease related metabolomic changes reflecting a biological microenvironment. However, metabolomic profiling it is not exclusive to fecal samples but also urine, intestinal tissue, plasma, serum as well as breath samples of patients with CD.[137] Finally, previous research has used FS in a similar way (e.g. differences in centrifugations) to perform immune cell stimulations assays.[138,237]

As all the experiments were performed *in vitro* (cell models: M2MQ and fibroblasts), and no literature discusses the concentration of FS, it is impossible to be entirely certain whether the dilution 1:1000 of FS used in the experiments (paper III) is **physiologically relevant**. However, the choice of this dilution was decided for different reasons described below. Depending on the specific research question we performed serial dilutions to establish a concentration curve from which the dilution was selected. For instance, in the case of the M2MQ experiments, the levels of secreted IL-10 served as reference point to

decide the dilution to use for FS (Figure 13). In a first experiment, high concentrations of 1:50 and 1:20 gave either undetected IL-10 levels (below limit of detection) or comparable levels as untreated cells (Figure 13, left). In addition, with these concentrations, by microscopical investigations cells looked either dead or "unhealthy" (no polarization, no proliferation). Concentration 1:1000 seemed preliminarily suitable as it reduced IL-10 levels compared to LPS control (Figure 13, left) and was investigated in a second experiment with more samples from CD and healthy (Figure 13, right). Testing of more diluted concentrations, 1:1000 compared to 1:500 and 1:3000 induced IL-10 in a concentration-dependent manner (Figure 13, right) and 1:1000 was chosen. Also, 1:1000 was previously shown to be able to influence M1MQs treated with FS from patients with UC.[139]

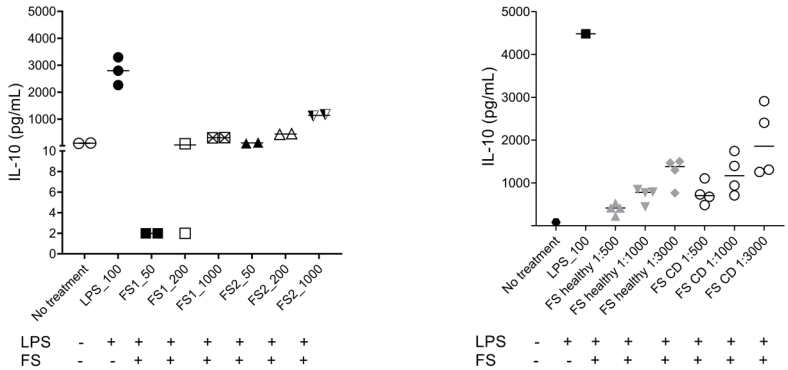


Figure 13. Establishing FS concentration to use for experiments with M2MQs. No treatment, refers to cells cultured with just culture media without LPS and without FS; LPS_100, cells cultured with LPS at a concentration 100 ng/mL without FS (this is also what we refer to as control in paper III); FS healthy, cells stimulated with FS from healthy subjects for 6 days and LPS for 1 day; FS CD, cells stimulated with FS from patients with CD for 6 days and LPS for 1 day.

3.6 GENE EXPRESSION

Gene expression analysis are widely performed to quantify gene expression in tissue or cells after RNA isolation and allows for detection of multiple genes or arrays with a small amount of sample. Although gene expression does not always reflect protein presence[238] or post-translational modification[239], it is a reliable indicator when it comes to basic research and more feasible than protein analysis when it comes to large sample sizes.

3.6.1 REVERSE TRANSCRIPTION - POLYMERASE CHAIN REACTION (RT-PCR)

RT-PCR[240] was performed in house to quantify human mRNA in whole mucosa, bulk IECs, LP cells, PBMCs and monocytes (paper I and II). Reverse transcription of mRNA into cDNA is performed as a proxy for the original mRNA. This is followed by amplification reactions using fluorescence and target primers and housekeeping genes. As the product adds up, the intensity of the fluorescence increases, hence the number of cycles required for the fluorescent signal to cross the threshold gives the Ct value (cycle threshold). The lower the Ct, the greater the amount of target mRNA in the sample; the Ct value is transformed and used for statistical testing. Regarding **housekeeping genes**, these are genes that are supposedly stable genes that do not change in health or disease. A synonym for housekeeping genes is reference genes. They are needed as controls to normalize the sample to that specific PCR cycle. Since they are stable genes in the cell and assumed to not change in health compared to disease they are used for normalization of the data as a first step of the calculations. In addition, they can be used as controls to check if the PCR reactions have worked. For IBD research, *RPLP0* and *GAPDH* genes are commonly used and do not change with inflammation or between individuals[241] (Figure 14). Nonetheless, despite one or two genes being traditionally selected as reference gene, the consensus in the field is that the use of multiple housekeeping genes should be employed.[242] The use of multiple housekeeping genes, specific for human intestinal tissue, would allow to have a more reliable normalization factor and detect small changes in gene expression, given that some genes that are expressed naturally low could be important to capture.

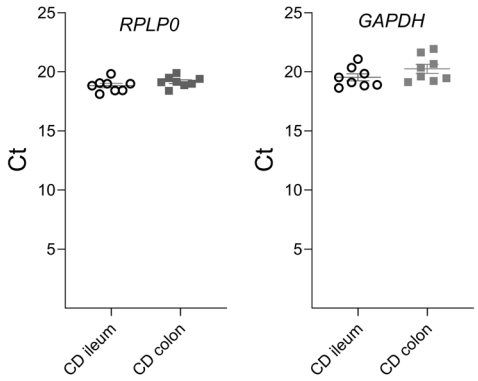


Figure 14: Stable gene expression of RPLP0 and GAPDH intestinal reference genes between tissue types and patients in CD. RPLP0, ribosomal protein lateral stalk subunit P0; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Ct, Cycle threshold.

3.6.2 PCR ARRAY

For quantifying gene expression in primary intestinal fibroblast cell lines CCD-18Co[243] I used the Fibrosis panel of RT^2 Profiler PCR Arrays (Qiagen)[244] in paper III. In contrast to traditional PCR, this method allows the simultaneous measurement of a panel of 84 genes for the same sample, includes multiple housekeeping genes, and several built in quality check parameters. An advantage of the method is that given the use of 384-well plates it is possible to run all genes for a single sample in one plate avoiding the intra plates variabilities that occurs in the traditional 96-well plates PCR. The gene panels are personalized, and the protocol is straightforward. A limitation of the method is that housekeeping genes are pre-selected to fit a wide range of cells/diseases and are not fibroblast- or CD-specific. This could possibly be solved by choosing a custom-made panel instead rather than a pre-made one.

3.6.3 mRNA COUNTS

Gene expression, by counts of the native mRNA, was performed using the nCounter Technology.[245] This analysis was performed in whole mucosa samples of patients with CD and control subjects (paper I and II), and on stimulated monocytes (paper II). As opposed to RT-PCR, with this method there is no amplification step. nCounter barcoding technology detects mRNA directly by using a capture and a reporter probe which are specific for the gene intended for measurement in the sample. Hence, it will build a mRNA-target-probe complex. After hybridization, the excess probes will be removed, and the purified complexes are immobilized on an imaging surface. Finally, each sample on the imaging surface is scanned by a mechanized fluorescence microscope, where labeled bar codes are directly counted, and the counts are used for further quantifications. An advantage of this method compared to the previous two is that it avoids the amplification step (cDNA synthetized from mRNA) and eliminating the technical variability that comes with that step. Another advantages are that it does not rely on having all the samples and genes on a single PCR plate and that it gives good results for formalin-fixed paraffin-embedded tissue samples. The samples are individually analyzed and reliably compared between each other. A disadvantage of the method is the amount of sample required and sometimes the existence of novel gene variants not captured in the pre-made panels.

3.7 SECRETED PROTEINS

A variety of targeted and untargeted protein/cytokine expression analyses are traditionally and increasingly being used in IBD to study the disease, as treatment biomarkers or in IBD pre-clinical research.[5,246,139] **Targeted methods** detecting either single or multiple proteins simultaneously allow the robust and quick quantification of key study proteins or large number of proteins if a semi-screening needs to be performed. In my thesis I have used various targeted methods for detection of proteins.

3.7.1 ENZYME LINKED IMMUNOSORBENT ASSAYS (ELISA)

ELISA was extensively used in my thesis work to detect main cytokines released from Mf, M2MQ[247] or that are relevant for CD.[248] More specifically, IL-1β and TNFα were measured in supernatants of cultured bulk LP tissue cells (named conditioned media in paper I and II); IL-10, TGFβ1 and TGFβ2 in supernatants of M2MQ; IL-6, MCP-1, IL-8, collagen and fibronectin in supernatants of intestinal fibroblasts (in paper III). All these proteins mentioned were reported in the respective papers, but also important to mention that several other proteins were measured to confirm that a method worked but were not reported. For instance, during the polarization of $CD14^+$ monocytes into M2MQ the cytokine IL-12, a cytokine prototypical of M1MQ and monocyte-derived dendritic cells, was confirmed to be low as expected.

3.7.2 PROXIMITY EXTENSION ASSAY (PEA)

PEA immunoassay, in the form of a commercial inflammation panel containing 92 proteins[249], was used for analyzing serum from patients with CD and healthy subjects (paper I), and supernatants of cultured bulk tissue cells from patient with CD and control subjects (paper II). PEA uses antibodies labelled with DNA-oligonucleotides probes that bind to target proteins in the test sample.[250] Once close, oligonucleotides hybridize and expand by DNA polymerase. This newly created piece of DNA barcode is amplified via traditional PCR. From Ct values, normalized protein expression is calculated that is inversed relative to the Ct value. An advantage of this method is the use of very little sample and after multiple freeze-thaw cycles of the samples the

proteins are still quantifiable.[251] Disadvantages of the method include the difficulty in comparing data from old samples with new samples, given possibly by differences in results due to batch-specific antibody performance.

3.7.3 ELECTROCHEMILUMINESCENCE ASSAY

This immunoassay using a commercial Mesoscale discovery (MSD)[252,253] panel was used to quantify proteins/cytokines specific for M2MQ (M-CSF, IL-1RA, TARC, IL-6, MCP-1, Eotoxin-2, IL-23, MDC, IL-13, and IL-4) in supernatants of FS-treated M2MQ (paper III). The method allows for targeted protein measurements in a small sample amount. First, each of the antibodies is combined with their linkers. A linker is a specific molecule binding to a specific location in the plate. Second, the test protein in the samples binds to the capture reagent and antibody-linker complex binds to the protein to form a final sandwich immunoassay. Finally, the signal is detected using electrochemiluminescence. Although it is a targeted approach, the method allows for a very high range of detection and sensitivity. Hence, it is advantageous for experiments, like this here, where due to a new stimulation condition of the cells there is no way to predict the concentration range of multiple proteins. A disadvantage of the method is the cost and the difficulty in directly comparing the results with other traditional methods such as ELISA.

3.8 FLOW CYTOMETRY

Flow cytometry is one of the most used techniques to quantify and identify human immune cells and, in numerous cases, non-immune cells. Flow cytometry is a method that provides rapid multi-protein detection, hence can distinguish cells for example in a tissue-derived cell suspension. The flow cytometry machine uses light sources to produce both scattered and fluorescent light signals that are transferred to detectors. Scattered lights (FSC, forward scatter and SSC, side scatter) are usually used to differentiate cells by size and granularity.[254] Fluorescent light comes from antibodies conjugated to fluorochromes and is used to distinguish cells that express that specific target for the antibody. The light and fluorescent signals are detected and transformed into electronic signals that are processed by a computer.

When using a panel, e.g. of 10 antibodies, we need to design the panel so that we detect all the fluorochromes (conjugated to an antibodies) without excessive spectral overlaps. For this purpose, antibodies are assigned fluorochromes that fall into different and distant spectra (especially when staining similar cell subsets) and antibodies specific for low expressed target proteins are assigned brighter fluorochromes to make sure they are detectable. Most spectral overlaps are removed by compensation while some spectral overlaps are acceptable for instance when the antibodies are used with the aim to exclude population of cells.[255] As the flow cytometry technique has a lot of background noise that can affect the results, controls are needed to distinguish background signal from real signal. Controls are also needed to make a correct *gating* (see below). The most commonly used controls are fluorescent minus one (FMO) or isotype controls. FMO is a panel of all antibodies in question but excluding the antibody of interest. This panel allows to visualize how the data looks without the antibody of interest. Isotype controls are antibodies of the same type and origin, conjugated to the same fluorochrome as the antibody of interest. Isotypes don't bind to the target cell marker and are used in parallel to set the gate.

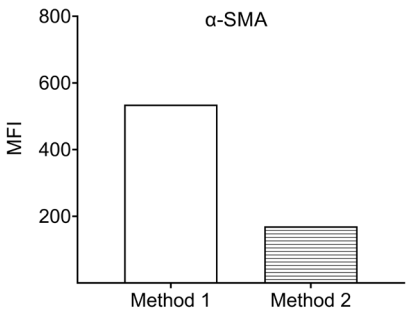


Figure 15. Expression of intracellular α-SMA protein on fibroblasts is affected by the method of staining. Method 1: staining of the extracellular proteins was performed first and was followed by the staining of intracellular protein α-SMA. Method 2: staining of intracellular protein α-SMA was performed first and was then followed by staining of extracellular proteins on the same sample of fibroblast cells. MFI, median fluorescence intensity.

In my thesis I have extensively used flow cytometry both in primary tissue cells as well as cell lines (Papers I, II, III). The technique I used allowed for up to 15 fluorochromes to stain for different **surface proteins** which gave a good characterization of many subsets of immune cell populations within a sample. After processing the intestinal tissue of patients with CD and controls to a cell suspension, samples were stained for surface markers. Regarding the M2MQ and fibroblast cell line, there was no need for pre-processing of the sample but it was performed just a detachment from the cell culture plate and then surface and/or intracellular protein staining.

Intracellular protein staining was performed for fibroblasts only. In contrast to performing surface staining alone, which was performed on live cells and was straightforward, the addition

of intracellular staining required some optimization. Intracellular staining includes extra steps such as fixation/permeabilization that can affect the fluorochrome-conjugated antibodies.[256] It is hence essential to ensure that the method used does not affect the expression of the protein of interest such as α-SMA in this case (see Figure 15 example).

Table 7. Surface and intracellular makers investigated by flow cytometry.

CD45	Used to identify all immune cells in the intestinal mucosa. Because it is a glycoprotein expressed by nucleated hematopoietic cells, it is estimated that up to 10% of an immune cell´s surface expresses this protein.[257]
CD3, CD19, CD56	Used to identify and exclude the lymphoid lineage (T cells, B cells and NK cells, respectively). As the research focus was on the myeloid lineage, we excluded the lymphoid lineage cells by these surface markers.[258]
HLA-DR	HLA-DR is an MHC class II surface receptor. MHC-II is an antigen-presenting molecule expressed at high levels on antigen presenting cells (dendritic cells, Mfs, B cells). With the previous exclusion of B cells HLA-DR identifies MNPs or dendritic cells and Mfs in the intestine.
CD14, CD11b, CD11c	Used to identify Mfs in the intestinal tissue. Subsets and Mf markers are described in the introduction 1.3.2
XCR1, CD1c, SIRPα	Used to identify dendritic cells.
CD14, CD163, CD206, CD36, CD11c	Used to characterize M2MQ polarization and effects of FS treatment. Included are prototypical markers for all Mfs, such as CD14, and markers under debate such as CD36.
EpCAM	Surface marker expressed by human IECs.[259] As digestion of each human sample is variable, this marker was used to distinguish the epithelial layer cells from the immune cells in the intestine. It is a widely used and reliable marker.
CD45, EpCAM	Absence of these two markers were used to delineate the stromal compartment of the intestine, which are non-immune, non-epithelial cells present in a lesser amount in the mucosa.[5, 260]

FAP, αSMA, PD-L1, CD21, TGFβ1	Surface and intracellular markers used to characterize the primary intestinal fibroblast cell line. Markers are either identification markers such as α-SMA, or markers related to intestinal fibrosis such as FAP.
Amine-reactive dye[261], 7-AAD	Used to identify dead cells
AnnexinV	Used to detect apoptotic granulocytes. Apoptotic granulocytes were assessed for phosphatidylserine translocation via AnnexinV binding.[262]
$CD14^+$, $CD66b^+$	Used for establishing the purity of MACS separated monocytes from PBMCs and granulocytes from blood respectively.

The table provides a short summary of all the flow cytometry markers used to identify and study the cell populations in this thesis: immune cells, fibroblasts, and IECs. CD, Cluster of differentiation; SIRPα, Signal regulatory protein α; EpCAM, Epithelial cell adhesion molecule (EpCAM); FAP, fibroblast activating protein; α-SMA, α-smooth muscle actin; PD-L1,Programmed death-ligand; TGFβ1, Transforming growth factor beta; 7-AAD, 7-aminoactinomycin D.

Box 2 – "Gating"

"*Gating*" in flow cytometry refers to the selection of sequential cell subsets for analysis to reach the subset of interest. Gating usually starts from a bulk/large mixture of different cell populations and goes down to specific targeted subsets. It is important for instance in the case of tissues given the huge heterogeneity of cell composition. Given that the digestion process of obtaining primary cell suspension decreases the number of viable cells, we also gate to exclude dead cells. Hence, gating usually starts with selecting cells (with FSC-Area/SSC-Area) to distinguish them from debris and continues into selecting singlets (by using FSC-Area/FSC-Hight or FSC-Area/FSC-Width), gating for live cells and finally cell subsets based on specific proteins. Gating is usually performed manually, and it needs controls (FMO), as well as knowledge of cell properties (e.g. what surface proteins and at what level they are expressed by a specific cell). One cannot start to analyze Mfs in flow cytometry without having knowledge of the basics of Mf identification in the tissue including their ontogeny. However, despite gating being widely used in research, there are substantial debates in how gates should be applied and efforts are being made to promote the use of automatic instead of manual gating.[263]

3.9 CELL SORTING

Cell sorting (or separation, isolation, purification) was performed in all the papers of this thesis for different purposes, but more frequently in paper III. The two methods below were used, which are also important methods existing in immunological research.

3.9.1 MAGNETIC-ACTIVATED CELL SEPARATION (MACS)

MACS is a method, developed by Miltenyi Biotec company[264], that uses magnetic nanoparticles conjugated to antibodies specific for a certain receptor/structure on a specific cell. MACS allows the separation/purification of a cell type of interest that is either retained (positive selection) or depleted (negative selection) when passing through a strong magnetic field. The main advantage is that the method is quick and easy to use, does not require a machine and can be used under sterile conditions in case the purified cells are intended for cell culture. The materials needed are only nanoparticle-conjugated antibodies, a magnetic separator, test tube or column and a washing buffer. Other advantages are that the procedure is gentle, and the cells do not undergo major stress. In addition, the yield and purity are good. The yield can be evaluated by simple cell counting but the purity requires the use of other methods such as FACS, however this is usually done only when a method is set up or a new kit is used. The main disadvantage is that it is less suitable for purifying low abundance populations of cells which can be purified at the expense of the yield. Furthermore, some studies have shown that the magnetic nanoparticles may hamper cell function.[265] In this thesis, MACS was used to positively select/isolate $CD14^+$ monocytes from PBMCs (Paper II and III) and to positively select/isolate $CD66b^+$ granulocytes from whole blood (Paper III). Both cell types were used for culturing and functional experiments. While isolation from PBMCs only required washing steps, isolation from whole blood required an extra step of red-blood cells elimination.

3.9.2 FLUORESCENT-ACTIVATED CELL SORTING (FACS)

FACS is a technique used to sort/isolate specific cell subpopulations based on phenotypes identified by flow cytometry.[266] A pool of a heterogeneous population of cells, e.g. from tissue, are stained with fluorescent antibodies and cells of interest are sorted, based on their antibody-fluorescent signal, into one or more cell subsets at a time. The advantages of the method are the possibility to sort even small subsets of cells, the simultaneous sorting of more than one type of cells from a sample and the yield/purity like MACS. The main disadvantages and differences compared to MACS are that it is a time-consuming procedure that requires setting up the machine in advance, and the cells can undergo mechanical stress due to the high stream speed in which the cells to be sorted pass through. In addition, there is a risk of two different types of contamination: (1) if the sorted cells are to be cultured, contamination from bacteria/particles can occur since the machine is not kept in sterile conditions; (2) since the cells are sorted into fluidic droplets following a stream there is risk that one type of cell can get contaminated with another type of cell. The second risk can be adjusted by decreasing the stream speed and changing the sorting mode but non-visible malfunctioning is also possible since the machine requires a lot of setting adjustments and experience. In this thesis, FACS was used to sort cells from intestinal mucosa cell suspensions: $CD45^+$ immune cells, $EpCAM^+$ IECs, $EpCAM^-CD45^-$ stromal cells, $CD14^+HLA\text{-}DR^+$ MNPs (paper I and II). All sorted cell types were used for RT-PCR gene expression analysis.

3.10 IMMUNOFLUORSCENCE (IF) MICROSCOPY

Multicolor immunofluorescence (IF) microscopy was employed to analyze the expression of membrane bound TREM-1 protein on formalin-fixed paraffine-embedded ileal mucosa from patients with CD and control subjects. The staining method used is called tyramide signaling amplification (TSA)[267, 268] and is an enzyme-mediated IF method that utilizes the catalytic properties of horseradish peroxidase to generate high-density labeling of a target protein *in situ*.[269] The method gives the advantage of simultaneously staining multiple proteins (up to 7) on the same tissue slide allowing a full image and comparison

of proteins on cells without the need to use different slides. Microwaving is used to quench endogenous peroxidase activity and to eliminate left-over antibodies after a target has been detected with a TSA-fluorophore. This IF protocol is both convenient when there is little tissue, and when there is the need to use multiple markers to identify a certain immune cell type (such as Mfs in our case). The disadvantage of the method is the initial laborious procedure when designing a panel and deciding the order of the proteins to stain for to be able to capture all the protein signals. For instance, lowly expressed proteins should be stained at the very end of the protocol to avoid the deterioration/fading by the microwaving/reagents used in the previous steps. Another limitation is that when it comes to Mfs not all markers, for instance used in flow cytometry, can be found to use in IF with TSA and this limits the choices. In paper II, IF was performed to simultaneously stain 5 proteins and localize the membrane-bound (1) TREM-1 protein *in* situ together with (2) CD163 (Mfs) (3) CD11c (Mfs/dendritic cells) (4) Pan-CK (IECs) and (5) DAPI (cell nuclei). The last two markers were used to delineate the tissue structure.

3.11 GRAPHICAL EXPERIMENTAL SETUPS

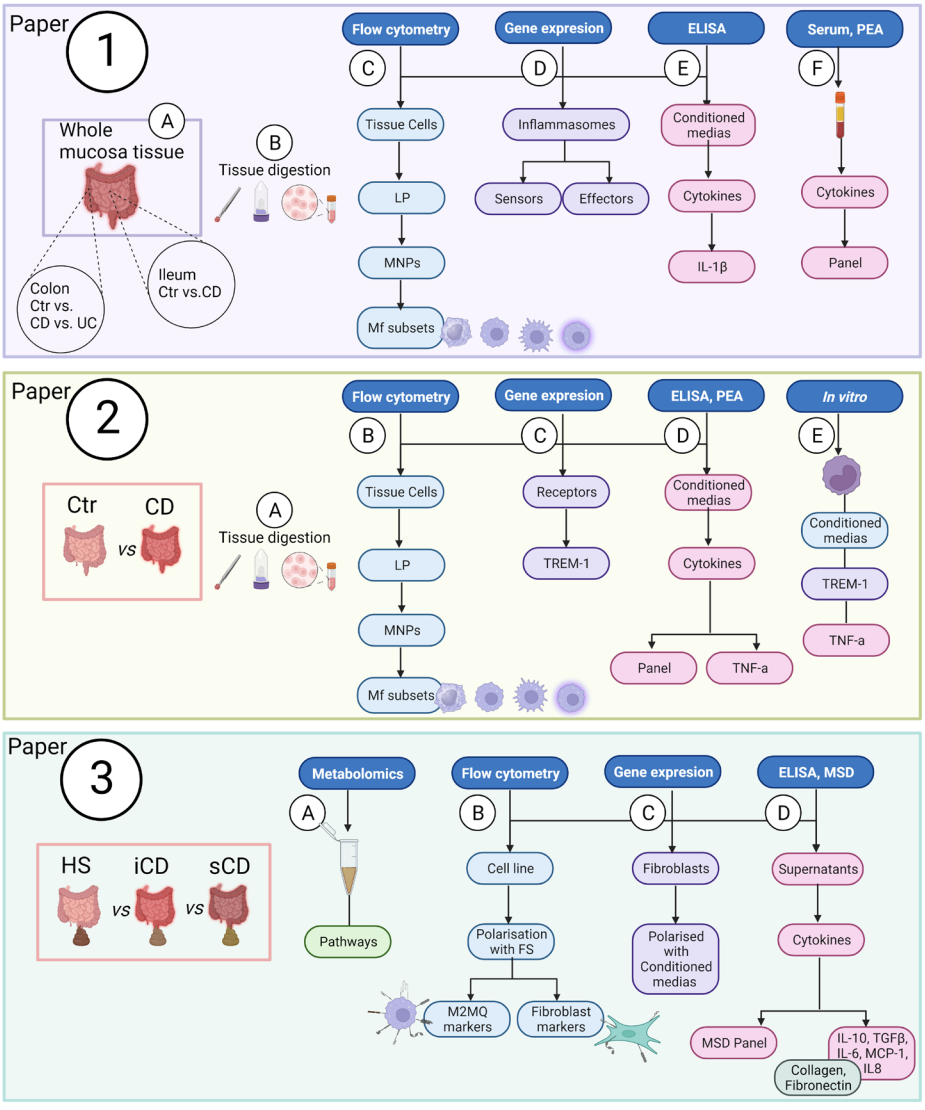


Figure 16. The figure illustrates a summary of the experiments and methods used in each paper of this thesis work. Clarifications: Paper I (D), Inflammasomes were analyzed in whole mucosa, bulk LP cells, bulk IECs as well as purified/sorted cells; Paper II (C), Gene expression analysis was performed on whole mucosa tissue of CD and UC as well as sorted cells of CD; Paper III (B-D), Fibroblasts similarly to M2MQ were analyzed with flow cytometry and ELISA.

4 RESULTS AND DISCUSSIONS

This chapter is a personalized summary and discussion of the main results, and a few aspects that for a matter of journalistic restrictions occupied less space in the respective papers. Despite the multiple findings reported in the respective papers, this section is intended to provide my point of view in relationship to innate immunity, macrophages, and CD disease-related parameters as the focus of this thesis.

4.1 PAPER I

Paper I[1] had a focus on inflammasome components expression, as innate immune platforms, in patients with CD. It was shown that gene expression of various inflammasome components, especially *NLRP3*, *MEFV* and *IL-1β*, were differentially expressed in intestinal samples of patients with CD ileitis, CD colitis (Figure 17) and patients with UC compared to controls. In addition, this differential expression was not only disease-related but also compartment-specific, given that there were also differences in expression between ileum and colon within CD. Finally, the gene relative expression of some

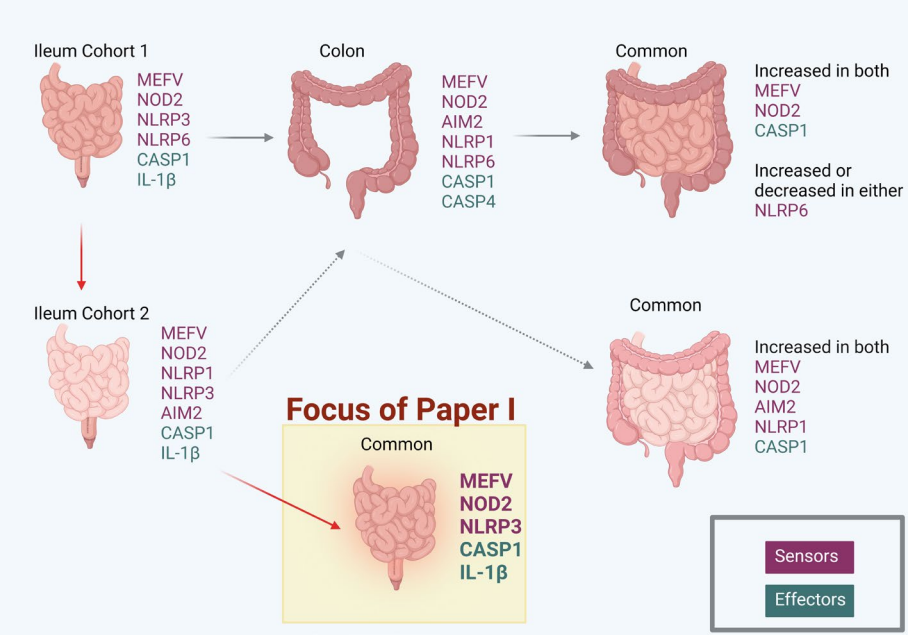


Figure 17. Results summary of Paper I (Figures 1 and 2 of the paper) showing gene expression changes in whole mucosa samples and with respect to compartment differences (ileum vs colon). Shown are only the inflammasome components that were differentially expressed between patients with CD and healthy/control subjects. Ileum cohort 1 and colon were analyzed with RT-PCR while ileum cohort 2 was analyzed with mRNA counts gene expression analysis. These results on whole mucosa analysis also directed the focus/continuation of the second part of the paper focusing only on MEFV, NLRP3, CASP1 and IL-1β, and only on ileum cohort 2.

inflammasome components correlated with the disease activity HBI (CD) and Mayo score (UC). Changes in gene expression possibly reflect abnormal signaling pathways driven by the inflammasome platform in the intestinal cells. Considering that the intestinal inflammasomes regulate responses to bacteria (commensal and pathogens)[270,271,182], and that during IBD there is an increased passage of bacteria and MAMPs, our results could point to an involvement of inflammasomes in disease pathogenesis. A complete understanding of the mechanisms driving the pathophysiology of ileal *vs* colonic CD, is currently under study.[47,272] As pointed out in the paper itself, ileal *vs* colonic CD may represent separate entities in terms of inflammasome activity and function which warrants further investigations.

Purified $CD14^{+}HLA\text{-}DR^{+}$ intestinal MNPs expressed relatively high levels of inflammasome genes *NLRP3*, *MEFV* and *IL-1β*. Among analyzed MNPs the expression of these genes was attributed to immature Mfs. As a confirmation, bulk LP cells secreted IL-1β protein, the main cytokine downstream inflammasome activation, that was also correlated with immature Mf frequency. In paper II we showed that among these MNPs, immature (pro-inflammatory) Mfs are increased in number in CD compared to controls.[2] The increase in number of immature Mfs together with the gene expression of inflammasome components indicates an increased activity of Mfs in the intestine of patients with CD that is driven partially by inflammasomes. Finally, a number of inflammasome genes, including *MEFV* and *NLRP3*, were also differentially expressed in unstimulated PBMCs of patients with CD compared to healthy subjects. PBMCs contain monocytes which are the precursors of immature Mfs in the intestine. A previous study has shown that LPS-activated PBMCs of patients with CD compared to controls had induced activation of *NLRP3* inflammasome.[58] MEFV levels in monocytes and monocyte-derived Mfs were also previously shown to regulate IL-1β release.[186] These suggest that blood immune cells, including monocytes, are predisposed to a higher inflammasome activity in CD compared to healthy before their arrival in the tissue due to the chronicity of CD. Whether the

differences in inflammasome expression in PBMCs we show are affected by cytokines/proteins serum of patients with CD providing different signals for priming of monocytes compared to healthy, or whether monocyte origin is shaped by the disease is yet to be established.

The CD cohort in this paper was a cohort of patients with active CD (defined by HBI) that due to various complications underwent surgery (at which timepoint the mucosal sample was also collected). Hypothesizing that this cohort would have a distinct systemic protein profile compared to a sex- and age-matched healthy cohort, we measured a panel of 92 inflammation-related serum proteins. Of these, 16 proteins were found differentially expressed including inflammatory cytokines such as IL-6, IL-8, CSF-1 (colony stimulating factor 1, also known as M-CSF) and CXCL1 (chemokine (C-X-C motif) ligand 1); and fibroblast growth factors such as FGF-19 and FGF-21. In addition, a number of these quantified serum proteins, correlated with inflammasomes gene expression in the LP, and belonged to pathways of macrophages/monocytes chemotaxis, responses to LPS and more. The differential expression of inflammatory proteins in CD serum such as CXCL1 and IL-6 have been previously confirmed by other studies in CD[273] and M-CSF spontaneously secreted by monocytes is believed to have a role in bringing active CD into remission.[274] Overall, the changes in serum protein levels described in this paper relate to the involvement of multiple cell types and suggest that circulating proteins reflect the chronicity of the ongoing processes within the GIT during active CD. Moreover, the correlation of some serum proteins and inflammasomes with the CD disease activity index support the prevailing knowledge of the active role of these inflammatory platforms in the disease pathogenesis.

4.2 PAPER II

In paper II,[2] the focus was on the role of the TREM-1 receptor in disease pathogenesis in patients with active CD. TREM-1 receptor is an amplifier of inflammation and the investigation in this paper attempted to block the receptor as potential CD treatment. We demonstrated that the TREM-1 receptor was induced in patients with CD and UC compared to controls, while no differences between ileal *vs* colonic expression in CD was detected. These finding are consistent with previous reports of increased TREM-1 expression in colonic mucosa[199], increase of TREM-1 peptidoglycan ligand[146,197] as well as increase

of soluble TREM-1[275], in IBD compared to healthy. Furthermore, our findings suggest only a tendency in correlation of TREM-1 expression with disease activity, measured by HBI, in active CD. As discussed in the introduction of this thesis, disease activity for CD is established by a vast number of index/scores/questionaries (that include or not an endoscopy) or by biochemical parameters (calprotectin, CRP). Whether the use of HBI alone, or any parameter alone, is a reliable measure for activity in CD is yet not clear. For instance, when it comes to soluble TREM-1 expression, previous studies have found that CRP was better correlated with soluble TREM-1 compared to the erythrocyte sedimentation rate[275], but both are biochemical parameters used to determine disease activity in patients with CD.

Mfs, among other effector cells, have been linked to the TREM-1 expression contribution in the intestinal mucosa. In paper II, TREM-1 receptor expression was differentially expressed in LP layer cells of patients with CD compared to controls, and within isolated subsets of LP cells TREM-1 gene expression was relatively higher in MNPs. This implied that TREM-1 expression was indirectly credited to MNPs for the most part. To start with, the main subsets of MNPs in the current cohort needed to be defined, according to the most up to date cell markers. As mentioned above, MNPs including Mfs subset functions are in continuous re-definition as the knowledge about the intestinal mucosa increases by e.g., the use of new single cell technologies that allow a broader mapping of subsets, or studies on specific cell markers. Given the new gating strategy for Mfs, as reported by the sophisticated work of Bujko *et al*[79] for healthy intestinal tissue, it made sense to apply these in CD compared to controls. A summary of flow cytometry results describing differences in MNP subsets, with focus on Mfs, are reported below (Table 8). Key findings included an increase in frequency in immature (monocyte-derived) Mfs (Table 8, row 3-5), which are distinguished from mature Mfs by CD11c expression. Mature Mfs did not change in frequency (Table 8, row 6-8). When assessing the TREM-1$^+$Mfs there was still an increase in CD compared to controls but that remained only among CD45$^+$ and not among Mfs (Table 8, row 16-18), suggesting that increase in TREM-1$^+$ cells is mainly due to the increase in number of immature newly-recruited Mfs (Table 8, row 17-18), rather than to mature Mfs. This has also been previously shown in mice.[276] Additionally, we confirmed TREM-1 expression on immature macrophages *in situ* by immunofluorescence analysis that showed a correlation between TREM-1 protein expression and a marker for immature Mfs, CD11c.[79] As abovementioned, CD11c is a marker that can also distinguish immature Mfs from mature Mfs.

Table 8. Summary of cell subsets changes reported in paper II[2] showing increase/decrease/no change in patients with CD compared to control subjects.

	Cell subsets changed in CD…	…among CD45$^+$	…among MNPs	…among Mfs	Paper[2] source
1	**MNPs**	Increase			Fig.2B
2	**Mfs**	Increase	Increase		Fig.2C, 2D
3	**Immature Mfs**	Increase			Fig. 2E
4	Mf1	Increase			Fig. 2I
5	Mf2	Increase			Fig. 2I
6	**Mature Mfs**	No change			Fig. 2F
7	Mf3	No change			Fig. 2J
8	Mf4	No change			Fig. 2J
9	**cDC**	Increase	No change		Suppl. 2F, Fig.2C
10	cDC1	No change			Suppl. 2G
11	cDC2	Increase			Suppl. 2G
12	**DN cDC**	No change			Suppl. 2G
13	**CD11$^-$CD14$^-$**	Decrease	Decrease		Fig.2C
14	**Lin$^-$**	No change			Suppl. 2E
15	**TREM-1$^+$**	Increase			Fig. 3A
16	**TREM-1$^+$Mfs**	Increase			Fig. 3B
17	TREM-1$^+$Mf1	Increase		No change	Fig. 3E
18	TREM-1$^+$Mf2	Increase		No change	Fig. 3E
19	TREM-1$^+$Mf3			No change	Fig. 3E
20	TREM-1$^+$Mf4			No change	Fig. 3E
21	**TREM-1$^+$CD11b$^+$** Granulocytes	Increase			Fig. 3B
22	**TREM-1$^+$cDCs**	No change			Fig. 3B

The gating levels are reported (among CD45$^+$, MNPs or Mfs). Lin$^-$ refers to non-lymphocytic cells as defined by CD3$^-$CD19$^-$CD56$^-$. The subset marked as CD11$^-$CD14$^-$ refers to plasmacytoid dendritic cells. CD45$^+$ refers to all immune cells within the LP layer. MNPs refers to the mononuclear phagocytes within the LP layer.

Given that the TREM-1$^+$ cells were mainly immature Mfs, which derive from newly-recruited monocytes, CD14$^+$ monocytes conditioned with LP cell supernatants from patients with CD or controls were used *in vitro* to mimic the

arrival of monocytes in the intestine and study the TREM-1$^+$ pathways. CD14$^+$ monocytes stimulated with either TREM-1 agonist, supernatants from LP-control or supernatants from LP-CD each showed a unique profile of gene expression when compared to the respective IgG controls. Furthermore, supernatants from LP-CD showed inflammation-related changes when compared with supernatants from LP-control, including increased levels of IL-6, IL-1β, CCL3 and CCL4. Finally, stimulation with TREM-1$^+$ agonist and stimulation with supernatants of LP-CD induced expression of several of the same genes, although at different expression levels. This can possibly be due to LP carrying ligands for TREM-1 as previously shown.[146]

Efforts were made to reduce immune activity induced by supernatants of LP-CD with a TREM-1 antagonist. Concomitant stimulation with supernatants from LP-CD and TREM-1 antagonist resulted in a decrease of relevant gene expression pathways such as pathogen response, cytokine and TLR signaling also including IL-6, CCL3 and CCL4 (these last three genes were previously increased with TREM-1 agonist treatment). However, the investigation of the three genes with a larger cohort of supernatants of LP-CD did not confirm the changes. This could be both due to the fact that TREM-1 is a receptor that could trigger various inflammatory signals and that supernatants of LP-CD have a vast number of ligands/cytokines/proteins that can interfere with the TREM-1 signaling in CD14$^+$ monocytes.[277,196,14]

Given that TREM-1 blockage alone did not result in predominant decrease of immune activity, a double blockage using TREM-1 and TNFα antagonist was investigated. The double blockage resulted in dampening of IL-6 on monocytes, slightly more pronounced as compared to anti-TNF alone. In paper I we showed that IL-6 protein expression levels are increased in the serum of CD and correlate with disease activity (HBI).[1] Similarly, other studies have shown serum IL-6, CRP levels and fecal calprotectin (different disease activity markers compared to the HBI used in our study) of patients with CD to be associated with clinical/endoscopic remission.[278] Mfs and CD4$^+$ T cells are the main producers of IL-6 in IBD and IL-6-driven polarization of Mfs into pro-inflammatory Mfs contributes to the inflammatory pool of IBD.[279,280] In addition, IL-6 action on the immune system is overall defined as pleiotropic and conditional on the phase of inflammation and targeted cell type.[14] However, dampening IL-6 in IBD concomitant treatment may has potential to provide clinical benefits.[44] TNFα antagonists are currently approved for CD management[281,282] but the drug works only for one-third of IBD patients[283]. It should be noted that, in our data, TNFα cytokine secreted from the LP cells of

CD consistently correlated with the immature Mf subpopulations and TREM-1^+ subpopulations. Further, patients with CD that showed high in TREM-1 expression also presented with high expression of TNFα. Hence, there might be a connection between the TREM-1 and TNF signaling. It is not clear if TREM-1^+ cells induce TNFα or if TNFα^+ cells are recruited consequently. As the TREM-1 receptor may join forces with other inflammation-related factors to influence the microenvironment of the CD mucosa concomitant blockage of more than one receptor might be more promising for dampening inflammation compared to a single drug.

4.3 PAPER I AND II

The inflammasome and TREM-1 papers used the same cohort of patients with active CD. The cohort was relatively small and patient heterogeneity is to be considered a limitation when investigating inflammation-related innate immune proteins. This is especially important because patients with active CD have as a main feature ongoing and chronic inflammation that is driven by innate immune cells. In addition to ongoing inflammation, there are many other complications, features or extraintestinal manifestations that define the CD disease complexity. It is widely shown that, even with stratification of large cohorts of patients with CD, it is challenging to establish disease biomarkers, predicting optimal treatment targets and methods to predict disease activity. In fact, as mentioned above, only one third of IBD patients respond to anti-TNF biological treatment, and the immunobiology of responders *vs* non-responders is still under discussion in the field. Patients with CD considered in paper I and II had mixed demographics, disease duration, treatment history and indications for surgery (complications). Despite this, no stratification was attempted because the cohort was relatively small. Although the use of small cohorts is not unique to our investigation but common in preclinical research in general, investigating a bigger cohort of patients with CD could reveal more concrete knowledge on the potential use of blocking TREM-1 and of the targets/roles of the inflammasomes in the pathogenesis of CD. Regarding the Mf subsets investigation, the small ileal cohort seemed to be enough and showed consistent results with literature in healthy intestine[79], mice[276] as well as CD studies in colon[105].

4.4 PAPER III

The focus of paper III was on M2MQ and intestinal fibroblast cells influence by feces-derived fecal supernatants (FS), as a model for intestinal microenvironment, and the communication with each other. FS were obtained from patients with active CD and compared to FS derived from healthy subjects for all experiments. In addition, patients with CD were subdivided into two categories: patients with stenosis (sCD) and patients without stenosis (iCD). Both subgroups had ongoing inflammation being a cohort of active CD. The M2MQ used are an *in vitro* model representing the tissue-resident Mfs in the intestine[284] given that the latter as such are less abundant in human intestinal tissue samples both for healthy and CD[65] and hard to obtain in sufficient numbers. The results showed that sustained priming for 3 days with CD-derived FS compared to healthy-derived FS, polarized $CD14^{+}CD163^{+}CD80^{-}$ M2MQs into a more anti-inflammatory phenotype. The anti-inflammatory capacity and polarization of M2MQ in this paper were defined by a series of proto-typical and exploratory surface and secreted proteins important for M2MQ function (summary of the results is shown in Figure 18).

In our results, CD-derived FS induced elevated levels of anti-inflammatory secretions (IL-10, TGFβ1, TGFβ2, IL-1RA, IL-6) and surface marker proteins (CD206, CD36, CD11c) compared to healthy-derived FS. Similarly, it was also previously shown for UC where UC-derived FS induced impaired polarization of M1MQ compared to healthy-derived FS.[139] Anti-inflammatory secretions, such as IL-10 and TGFβ1, are prototypical of tissue-resident Mfs.[143] In addition, tissue-resident Mfs, especially $CD206^{high}$ Mfs, secrete high levels of IL-10 and are involved in mucosal healing.[285,108] Regarding CD36, functions have been related to efferocytosis of macrophages[115], absorption of lipids in the intestine[117], and can influence macrophage response to bacterial LPS[286]. In mice, CD36-deficient Mfs were shown to result in reduced kidney fibrosis compared to wild-type, and this effect was attributed to their efferocytosis function.[115] An increase of CD36, by FS from iCD and sCD compared to healthy in our study, might indicate that FS of sCD contains increased signals to perform efferocytosis during fibrosis. However, the efferocytotic capacity of M2MQ toward apoptotic primary neutrophils was tested, and FS was not able to affect efferocytosis in our *in vitro* setting. Given that FS in our study was added to the cell culture in an attempt to mimic the intestinal microenvironment of CD, the changes induced by FS could point to the efforts of tissue-resident Mfs to try to stabilize pro-inflammatory processes through

FS, and restore wound healing. Given the scarcity of treatments for CD, with current treatments mainly holding off the flares with immunosuppression, promoting mucosal healing has been proposed as a promising therapeutic approach to direct new studies treating CD.[287]

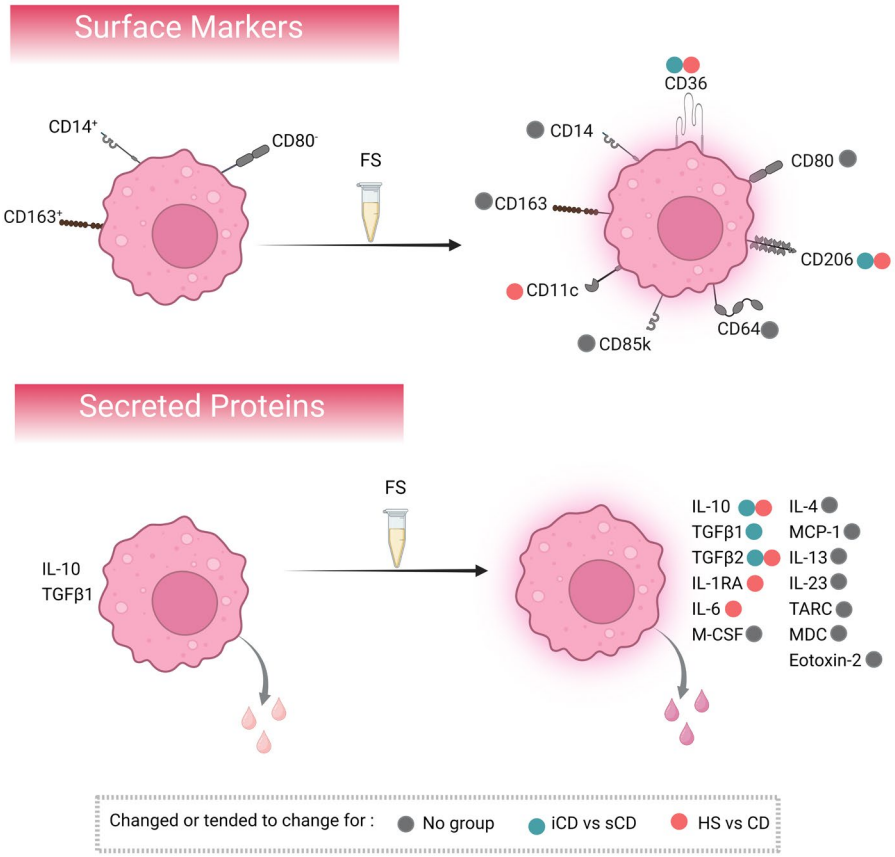


Figure 18. Surface markers and secreted proteins by M2MQ stimulated with FS from CD, iCD, sCD and healthy subjects. On the left, the markers/proteins that were expected to be expressed from the M2MQ polarization method are shown. On the right, the markers/proteins that were aimed to investigate the effects of FS on M2MQ are shown. CD(n), Cluster of Differentiation (number); IL, Interleukin; TGFβ, Transforming growth factor beta; M-CSF, Macrophage-colony stimulating factor; MCP-1, Monocyte chemoattractant protein-1; TARC, Thymus and activation regulated chemokine or CCL17; MDC, Macrophage-derived chemokine.

Stenosis in patient with CD, as a complication occurring in one out of three patients within 10 years of diagnosis, presents as chronic transmural inflammation with subsequent hyperplasia and fibrosis buildup.[288] Tissue-resident Mfs are found in and around fibrotic areas.[164] In our investigation the majority of differences, induced by FS on M2MQ, were observed for the

comparison CD *vs* healthy subjects, although a few differences were evident for the comparison iCD *vs* sCD as well (Figure 18). Upon priming the M2MQ with FS from patients with sCD compared to iCD, there was an upregulation of surface markers CD206, CD36 and a slight separation by the total secreted protein profiles measured with MSD. Regarding CD206, $CD206^+$ Mfs are described in literature as pro-fibrotic and found on fibrotic areas locations in the intestine.[289,290,291] Interestingly, CD206 seems also to be related to IL-10 as colonic Mfs expressing CD206 seem to acquire higher expression of IL-10.[108] Despite an increase of CD206 in our data it is not clear from literature the precise type of involvement in the fibrosis processes of CD206 and whether (1) $CD206^+$ Mfs are recruited following initial pro-fibrotic signals, or if (2) $CD206^+$ Mfs themselves contribute to the initiation of fibrosis buildup, or (3) if CD206 Mfs are found in fibrotic areas given the tissue structure changes (e.g. stenosis, strictures). A translation of our *in vitro* findings to the *in vivo* setting requires further investigations.

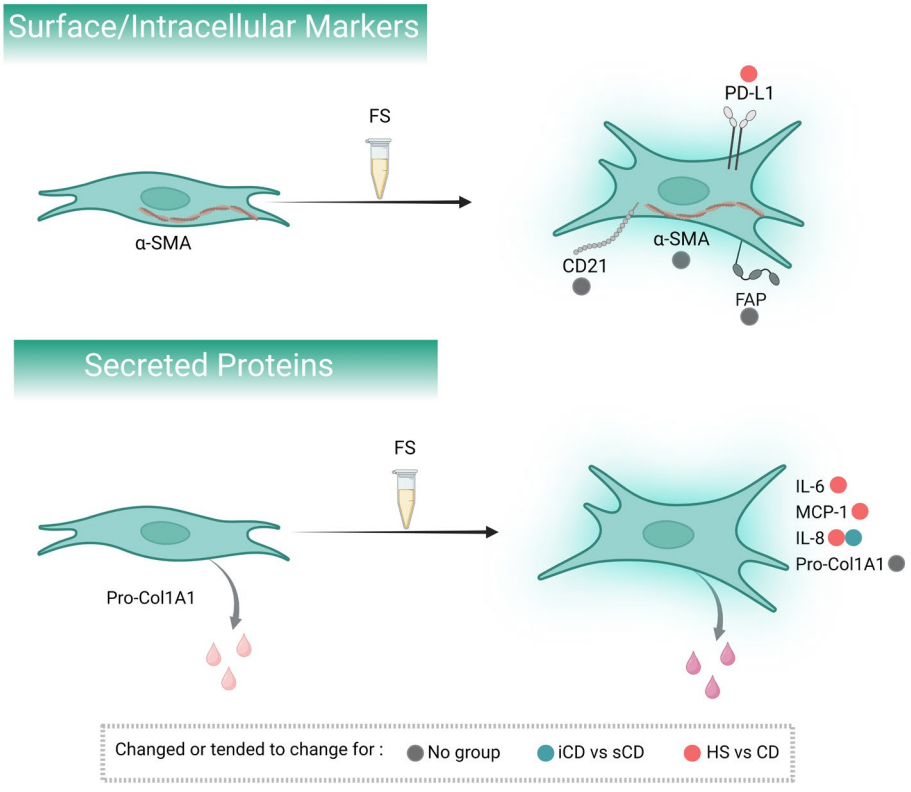


Figure 19. Surface/intracellular markers and secreted proteins by fibroblasts stimulated with FS from CD, iCD, sCD and healthy subjects. On the left, the markers/proteins that were expected to be expressed from the fibroblast culture method used are shown. On the right, the markers/proteins that were aimed to investigate the effects of FS on fibroblasts are shown. α-SMA, alfa-Smooth muscle actine; CD(n), Cluster of Differentiation (number); FAP, Fibroblast activating protein; PD-L1, Programed death-ligand; Pro-Col1A1, Pro-collagen I alpha 1; IL, Interleukin; MCP-1, Monocyte chemoattractant protein-1.

Fibrosis is most often defined as fibroblast/myofibroblast proliferation and deposition of excessive extracellular matrix proteins (for instance collagen) that causes hardening of the tissue.[158] However, in CD fibrosis is not standalone but is the end result of chronic inflammatory responses, hence the two processes are believed to be concurrent and co-dependent of each other.[292] In paper III, a primary intestinal fibroblast cell line was stimulated with FS to mimic the effects the CD microenvironment has on fibroblasts (the key player cells of fibrosis). CD-derived FS compared to healthy-derived FS induced pro-inflammatory secretions (IL-6 and MCP-1) in intestinal fibroblasts (summary of the results is shown in Figure 19). It has been shown that fibroblasts in CD express both IL-6, MCP-1[104] and CD colonic fibroblasts stimulated with LPS produce increased levels of IL-6[293]. This goes hand-in-hand with the fact that, as immune-like cells, fibroblasts are able to respond to pathogens and also express TLRs and detect MAMPs.[294] A pro-inflammatory involvement of fibroblasts in response to FS suggests that, as sentinel cells, fibroblasts are affected differently by the microenvironment in patients with CD compared to healthy subjects.

Furthermore, expression of phenotypical and functional markers, such as α-SMA, FAP and collagen, were not affected by FS *per se* in the present study (Figure 19). Our finding is consistent with previous studies that have shown that expression of these three proteins occur simultaneously in the tissue.[295,165] In addition, FAP-expressing fibroblasts are found in fibrotic lesions.[164,165] The observation of no change for either of these proteins could imply that FS does not have a direct effect on fibroblast phenotype (α-SMA, FAP) and function related to tissue structure/fibrosis such as extracellular matrix formation (collagen). However, our *in vitro* cell model was to start with α-SMA^{high}, which represents the myofibroblasts phenotype in the tissue itself, hence this requires further investigations.

Finally, paper III aimed to mimic Mf-fibroblast interactions by stimulating fibroblasts with FS-primed M2MQ conditioned medias. Because conditioned media of M2MQs itself contain cytokines/inflammatory proteins, it was not possible to analyze these secreted proteins from fibroblasts stimulated with conditioned medias, as we would not be able to attribute the detection uniquely to fibroblasts. The analyses were therefore focused on cellular measurements (surface/intracellular markers and gene expression) (summary of the results is shown in Figure 20).

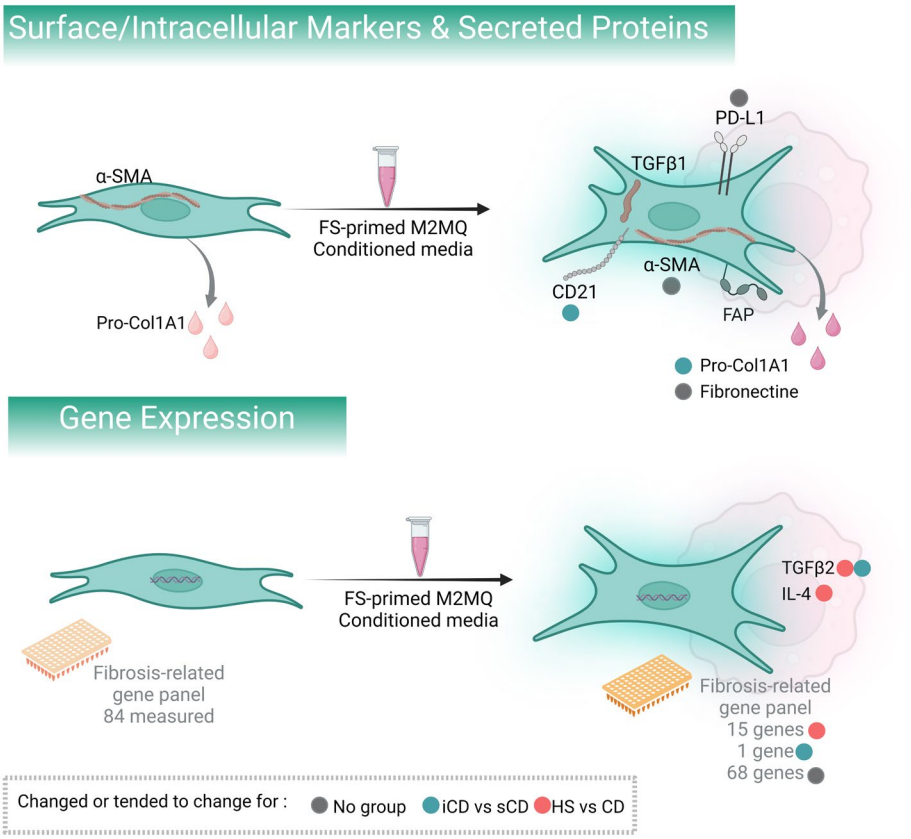


Figure 20. Surface/intracellular markers, secreted proteins and gene expression of fibroblasts stimulated with FS-primed M2MQ conditioned media from CD, iCD, sCD and healthy subjects. On the left, the expressed markers/proteins that were expected/measured from the fibroblast culture method used are shown. On the right, the markers/proteins that were aimed to investigate are shown. α-SMA, alfa Smooth muscle actine; Pro-Col1A1, Pro-collagen I alpha 1; CD(n), Cluster of Differentiation (number); FAP, Fibroblast activating protein; PD-L1, Programed death-ligand; TGFβ, Transforming growth factor beta; IL, Interleukin.

In patients with CD compared to healthy subjects, there were no changes in surface/intracellular markers prototypical of fibroblasts phenotype/fibrosis (α-SMA, FAP) or extracellular matrix proteins (collagen, fibronectin) (Figure 20). Albeit, following gene expression analysis, a slight altered expression of 15 genes were observed. These genes belonged to e.g the TGFβ superfamily (e.g. TGFβ2) or pro-fibrotic genes (e.g. IL-4) among others (Figure 20). Of note, TGFβ2 gene expression was the only gene altered for both comparison CD *vs* HS and iCD *vs* sCD. It has been previously shown that TGFβ2 expression is increased in active IBD[296] and knocking out TGFβ2 decreases liver fibrosis in mice[297]. Nevertheless, signaling through the TGFβ superfamily members is multifactorial in relationship to fibrosis and inflammation on fibroblasts, and TGFβ2 is even less reported in literature compared to TGFβ1 for the intestinal tissue.[298] The meaning of these preliminary results require further investigations. As the dynamics of Mf-fibroblast interactions in the fibrotic areas are unclear, and anyway tissue-specific, other studies have tried to simulate the wound healing process by stimulating fibroblasts with Mf-conditioned media.[299,300] However in these studies, Mf-conditioned media were produced either with LPS induced M0/M1MQs/M2MQs polarisaiton[299] or conditioned media of Mfs treated with anti-inflammatory drugs[300]. In addition, previous research with M2MQ-conditioned media have not shown to promote profibrotic responses in lung fibroblasts,[301] consistent with our data where no changes in collagen expression were observed for CD *vs* healthy subjects. Although, to our knowledge no previous study has been performed with intestinal conditioned media with FS addition, neither in health nor disease, our suggestive data on TGFβ and IL-4 merits further investigations to clarify their involvement.

To summarize, fecal supernatants obtained from patients with CD show a differential polarization of M2MQ, via anti-inflammatory proteins such as IL-10 after LPS activation, while efferocytosis function is not affected. Furthermore, upon addition of FS, intestinal fibroblasts induced pro-inflammatory secretions, such as IL-6, but overall, very little fibrosis related changes induced by FS were observed in CD compared to healthy subjects. Finally, fibroblasts stimulated with FS-primed M2MQs conditioned medias from CD showed changes in TFGβ superfamily and IL-4 gene expression, among others. Overall, although both CD *vs* healthy and iCD *vs* sCD were compared for all analysis, most significant changes were observed for CD compared to healthy subjects, alluding to the fact that the microenvironment of CD is more similar within subgroups than in comparison to healthy.

5 CONCLUSIONS AND FUTURE STANDPOINTS

5.1 PAPER I

Paper I provided further support of the significance of NLRP3 and MEFV inflammasomes in CD pathogenesis. These were shown through gene expression analysis of patients with active CD and relevant cell types (LP, purified MNPs, Mfs, PBMCs). Furthermore, as NLRP3 and MEFV inflammasomes expression was attributed to immature mucosal Mfs and PBMCs, the paper proposes that the high influxes of monocytes recruited to the inflammatory site during CD may be pre-programmed already in blood for altered inflammasome function when polarized in the intestine into immature Mfs. In addition, a differential expression of inflammatory serum proteins of patients with active CD patients from healthy age-matched subjects also correlate to expression of some inflammasome genes at the mucosal level.

Globally, research of the expression and role of inflammasomes in patients with active CD patients can provide knowledge to design better treatments intended to target the intestinal innate immune system. Given the heterogeneity of patients within the CD cohort regarding inflammasome activity, it would be ideal to also identify patient subgroups that could respond to inflammasome treatments. Could those with high disease activity index be a target group? Potential targets for treatments could include IL-1β inhibition with the aim to block a broader inflammasome range or a specific sensor blocking, such as NLRP3, with the final aim to control amplification of inflammation based on one inflammasome complex.

One might wonder why intestinal inflammasomes are not targeted to treat intestinal diseases already? Since the discovery of “The inflammasomes” in 2002,[168] targeting is not yet a reality as there are still a lot of knowledge gaps in literature. Just to mention four future perspectives. First, physiological ligands that trigger the inflammasome sensors *in vivo*, in healthy human intestine, are not entirely known, and it is not known how these ligands are different in the diseased intestine given the massive inflammatory changes. For example, for NLRP3, the most studied inflammasome, Nigericin is experimentally used to trigger the sensor, however nigericin is not a

physiological ligand. Second, inflammasome activation is cell-specific and tissue-specific, in addition to being location-specific (e.g. ileum *vs* colon) and disease-specific (e.g. CD *vs* UC). Third, to add to this complexity, inflammasomes use different downstream effector pathways and all these pathways must be considered in a patient-disease-tissue-cell-specific manner for future studies. Fourth, since it is known that inflammasomes play a physiological role in health, targeting them during disease might potentially mean affecting their protective function as well. Is their activation in the disease higher because they are being used as a protective mechanism in the disease? Consideration should be placed into the physiological innate immune role of inflammasomes with the final aim to successfully target them.

5.2 PAPER II

Paper II showed in summary that TREM-1^{+} Mfs increase in the mucosa of patients with active CD compared to non-inflamed controls. The increase in Mfs largely explains the increase of the TREM-1 receptor expression at the gene and protein level from the same patients in whole mucosa. Stimulation of monocytes with TREM-1 agonist/antagonist, supernatants from LP (CD and control), to mimic the arrival of monocytes in the intestine, showed unique inflammatory pathways influencing TREM-1 signaling. Finally, the attempt to block TREM-1 concurrent with anti-TNF, to stop amplification of inflammation, showed a decrease of IL-6 secretion that warrants further investigation.

Overall, the data suggest that there is an increase in intestinal mucosal immature Mfs in patients with active CD compared to controls, and high expression of the TREM-1 receptor, attributed to immature Mfs in with CD is linked to damage and chronic inflammation. Future studies should investigate further blocking the TREM-1 receptor with or without anti-TNF treatment in different scenarios and with a larger sample size, or in patients with TREM-1^{high} expression who stop responding to conventional therapies.

Studies in CD with patient material and TREM-1 are only a few. There is still a debate on the ligand repertoire and signaling of TREM-1 even for diseases in which TREM-1 is more stablished (such as sepsis). In the future, investigating a larger patient pool, with the possibility of detailed patient

stratification, could clarify the prospective use of anti-TREM1 as treatment or enhancement or other therapies.

5.3 PAPER III

Paper III results suggested that Mfs and fibroblasts are affected by the fecal microenvironment signals, to alter inflammation-related secretions in patients with CD compared to healthy subjects. Fecal-derived factors (FS) in CD cause an exaggerated anti-inflammatory action of tissue-resident Mfs and pro-inflammatory effects on fibroblasts that, despite being opposite effects, align with the cell function in the tissue (e.g. M2MQ are naturally primed to be anti-inflammatory). Furthermore, the results also lead to the conclusion that the role of intestinal fibroblasts in chronic inflammation in active CD goes beyond tissue structure, both for inflammatory CD with or without stenosis. Together these give confidence that inducing changes, e.g. through treatments improving the microbiota, the anti-inflammatory effects of tissue-resident Mfs, and promoting resolution and wound healing, could be promising to find feasible treatments for patients with CD.

Compared to tissue-resident Mfs in the intestine, monocyte-derived M2MQs will differ as they lack the full environmental signals and ontogeny. Single-cell studies could be employed to consider low abundant tissue-resident cells in the intestine to allow studying their function. Likewise, the use of a primary intestinal fibroblast cell lines ought to in the future be replaced with patient-derived fibroblasts. Despite the *in vitro* microenvironment of all our cell models being an attempt to mimic the *in vivo* setting, this supports that future studies targeting the microbiota could be promising to counteract the chronicity of CD even at a pre-stenotic stage, hopefully in parallel with the development of clinically established pre-fibrosis biomarkers.

Forthcoming studies in the field should make sure to supply the current lack of *in vitro*, *ex vivo* and animal models to study fibroblast polarization and fibrosis, and these studies should consider the fact that fibrosis is a long-term process which is impossible to capture in animal studies. In the future, larger cohorts with higher number of patients should be employed to also include patients with only stenosis (fibrosis) which was not included in the current paper, with the final aim to find personalized treatments for patients with CD.

REFERENCES

1. Gorreja, F. *et al.* MEFV and NLRP3 Inflammasome Expression Is Attributed to Immature Macrophages and Correlates with Serum Inflammatory Proteins in Crohn´s Disease Patients. *Inflammation* **45**, 1631–1650 (2022).
2. Caër, C. *et al.* TREM-1+ Macrophages Define a Pathogenic Cell Subset in the Intestine of Crohn's Disease Patients. *J. Crohns Colitis* (2021) doi:10.1093/ecco-jcc/jjab022.
3. Gorreja, F., Rush, S. T., Kasper, D. L., Meng, D. & Walker, W. A. The developmentally regulated fetal enterocyte gene, *ZP4* , mediates anti-inflammation by the symbiotic bacterial surface factor polysaccharide A on *Bacteroides fragilis*. *Am. J. Physiol.-Gastrointest. Liver Physiol.* **317**, G398–G407 (2019).
4. Gorreja, F. & Walker, W. A. The potential role of adherence factors in probiotic function in the gastrointestinal tract of adults and pediatrics: a narrative review of experimental and human studies. *Gut Microbes* **14**, 2149214 (2022).
5. Alfredsson, J. *et al.* Isobaric labeling-based quantitative proteomics of FACS-purified immune cells and epithelial cells from the intestine of Crohn's disease patients reveals proteome changes of potential importance in disease pathogenesis. *PROTEOMICS* **23**, 2200366 (2023).
6. Valido, E. *et al.* Systematic Review of Human and Animal Evidence on the Role of Buckwheat Consumption on Gastrointestinal Health. *Nutrients* **15**, 1 (2022).
7. Gorreja, F. Gene expression changes as predictors of the immune-modulatory effects of probiotics: Towards a better understanding of strain-disease specific interactions. *NFS J.* **14–15**, 1–5 (2019).
8. Callahan, D. The WHO Definition of 'Health'. *Hastings Cent. Stud.* **1**, 77 (1973).
9. OxfordLanguages. https://languages.oup.com/google-dictionary-en/ (2023).
10. Doran, A. C., Yurdagul, A. & Tabas, I. Efferocytosis in health and disease. *Nat. Rev. Immunol.* **20**, 254–267 (2020).
11. Mehrotra, P. & Ravichandran, K. S. Drugging the efferocytosis process: concepts and opportunities. *Nat. Rev. Drug Discov.* **21**, 601–620 (2022).
12. Ziegler-Heitbrock, L. *et al.* Nomenclature of monocytes and dendritic cells in blood. *Blood* **116**, e74–e80 (2010).
13. Li, H. *et al.* The outer mucus layer hosts a distinct intestinal microbial niche. *Nat. Commun.* **6**, (2015).
14. Friedrich, M., Pohin, M. & Powrie, F. Cytokine Networks in the Pathophysiology of Inflammatory Bowel Disease. *Immunity* **50**, 992–1006 (2019).
15. Mowat, A. M. & Agace, W. W. Regional specialization within the intestinal immune system. *Nat. Rev. Immunol.* **14**, 667–685 (2014).

16. Okumura, R. & Takeda, K. Roles of intestinal epithelial cells in the maintenance of gut homeostasis. *Exp. Mol. Med.* **49**, e338–e338 (2017).
17. Chalkidi, N., Paraskeva, C. & Koliaraki, V. Fibroblasts in intestinal homeostasis, damage, and repair. *Front. Immunol.* **13**, 924866 (2022).
18. Valatas, V., Kitamura, K., Ward, S. G. & Kolios, G. Editorial: Stromal and immune cell interactions in intestinal inflammation and fibrosis. *Front. Immunol.* **14**, 1152140 (2023).
19. Sun, H., Tan, J., Chen, H., Wu, N. & Su, B. Immune niches orchestrated by intestinal mesenchymal stromal cells lining the crypt-villus. *Front. Immunol.* **13**, 1057932 (2022).
20. Jasso, G. J. *et al.* Colon stroma mediates an inflammation-driven fibroblastic response controlling matrix remodeling and healing. *PLOS Biol.* **20**, e3001532 (2022).
21. IBDMDB Investigators *et al.* Multi-omics of the gut microbial ecosystem in inflammatory bowel diseases. *Nature* **569**, 655–662 (2019).
22. Alatab, S. *et al.* The global, regional, and national burden of inflammatory bowel disease in 195 countries and territories, 1990–2017: a systematic analysis for the Global Burden of Disease Study 2017. *Lancet Gastroenterol. Hepatol.* **5**, 17–30 (2020).
23. Ananthakrishnan, A. N. Epidemiology and risk factors for IBD. *Nat. Rev. Gastroenterol. Hepatol.* **12**, 205–217 (2015).
24. de Souza, H. S. P. & Fiocchi, C. Immunopathogenesis of IBD: current state of the art. *Nat. Rev. Gastroenterol. Hepatol.* **13**, 13–27 (2016).
25. Dige, A. *et al.* Effects of Anti-TNF *α* Treatment on Mucosal Expression of IL-17A, IL-21, and IL-22 and Cytokine-Producing T Cell Subsets in Crohn's Disease. *Mediators Inflamm.* **2018**, 1–7 (2018).
26. Mayo, C. Crohn´s disease. https://www.mayoclinic.org/diseases-conditions/crohns-disease/symptoms-causes/syc-20353304 (2022).
27. Rogler, G., Singh, A., Kavanaugh, A. & Rubin, D. T. Extraintestinal Manifestations of Inflammatory Bowel Disease: Current Concepts, Treatment, and Implications for Disease Management. *Gastroenterology* **161**, 1118–1132 (2021).
28. Thia, K. T., Sandborn, W. J., Harmsen, W. S., Zinsmeister, A. R. & Loftus, E. V. Risk Factors Associated With Progression to Intestinal Complications of Crohn's Disease in a Population-Based Cohort. *Gastroenterology* **139**, 1147–1155 (2010).
29. Mary, J. Y. & Modigliani, R. Development and validation of an endoscopic index of the severity for Crohn's disease: a prospective multicentre study. Groupe d'Etudes Therapeutiques des Affections Inflammatoires du Tube Digestif (GETAID). *Gut* **30**, 983–989 (1989).
30. Khanna, R. *et al.* Endoscopic scoring indices for evaluation of disease activity in Crohn's disease. *Cochrane Database Syst. Rev.* **2016**, (2016).
31. Harvey, R. F. & Bradshaw, J. M. A SIMPLE INDEX OF CROHN'S-DISEASE ACTIVITY. *The Lancet* **315**, 514 (1980).

32. Vermeire, S., Schreiber, S., Sandborn, W. J., Dubois, C. & Rutgeerts, P. Correlation Between the Crohn's Disease Activity and Harvey–Bradshaw Indices in Assessing Crohn's Disease Severity. *Clin. Gastroenterol. Hepatol.* **8**, 357–363 (2010).
33. Yang, D.-H. *et al.* Usefulness of C-Reactive Protein as a Disease Activity Marker in Crohn's Disease according to the Location of Disease. *Gut Liver* **9**, 80–86 (2015).
34. Zittan, E., Kelly, O. B., Gralnek, I. M., Silverberg, M. S. & Hillary Steinhart, A. Fecal calprotectin correlates with active colonic inflammatory bowel disease but not with small intestinal Crohn's disease activity: Fecal Calprotectin and disease location in IBD. *JGH Open* **2**, 201–206 (2018).
35. Lundström, M. L. *et al.* Faecal biomarkers of neutrophil and eosinophil origin reflect the response to biological therapy and corticosteroids in patients with IBD. *Clin. Transl. Gastroenterol.* **Publish Ahead of Print**, (2023).
36. Freitas, M. *et al.* Ultrasonographic scores for ileal Crohn's disease assessment: Better, worse or the same as contrast-enhanced ultrasound? *BMC Gastroenterol.* **22**, 252 (2022).
37. Roseira, J., Ventosa, A. R., Sousa, H. T. & Brito, J. The new simplified MARIA score applies beyond clinical trials: A suitable clinical practice tool for Crohn's disease that parallels a simple endoscopic index and fecal calprotectin. *United Eur. Gastroenterol. J.* **8**, 1208–1216 (2020).
38. Mazor, Y. *et al.* Prediction of disease complication occurrence in Crohn's disease using phenotype and genotype parameters at diagnosis. *J. Crohns Colitis* **5**, 592–597 (2011).
39. Lin, X. *et al.* Intestinal strictures in Crohn's disease: a 2021 update. *Ther. Adv. Gastroenterol.* **15**, 175628482211049 (2022).
40. Ashton, J. J. *et al.* Prediction of Crohn's Disease Stricturing Phenotype Using a *NOD2*- derived Genomic Biomarker. *Inflamm. Bowel Dis.* **29**, 511–521 (2023).
41. Bourgonje, A. R. *et al.* Serological biomarkers of type I, III and IV collagen turnover are associated with the presence and future progression of stricturing and penetrating Crohn's disease. *Aliment. Pharmacol. Ther.* **56**, 675–693 (2022).
42. Hegarty, L. M., Jones, G.-R. & Bain, C. C. Macrophages in intestinal homeostasis and inflammatory bowel disease. *Nat. Rev. Gastroenterol. Hepatol.* **20**, 538–553 (2023).
43. Pariente, B. *et al.* Validation and Update of the Lémann Index to Measure Cumulative Structural Bowel Damage in Crohn's Disease. *Gastroenterology* **161**, 853-864.e13 (2021).
44. Neurath, M. F. Targeting immune cell circuits and trafficking in inflammatory bowel disease. *Nat. Immunol.* **20**, 970–979 (2019).

45. van der Post, S. *et al.* Structural weakening of the colonic mucus barrier is an early event in ulcerative colitis pathogenesis. *Gut* **68**, 2142–2151 (2019).
46. Mitsialis, V. *et al.* Single-Cell Analyses of Colon and Blood Reveal Distinct Immune Cell Signatures of Ulcerative Colitis and Crohn's Disease. *Gastroenterology* **159**, 591-608.e10 (2020).
47. Chang, J. T. Pathophysiology of Inflammatory Bowel Diseases. *N. Engl. J. Med.* **383**, 2652–2664 (2020).
48. Andersson, E. *et al.* Subphenotypes of inflammatory bowel disease are characterized by specific serum protein profiles. *PLOS ONE* **12**, e0186142 (2017).
49. The International IBD Genetics Consortium (IIBDGC) *et al.* Host–microbe interactions have shaped the genetic architecture of inflammatory bowel disease. *Nature* **491**, 119–124 (2012).
50. Watanabe, T., Kitani, A. & Strober, W. NOD2 regulation of Toll-like receptor responses and the pathogenesis of Crohn's disease. *Gut* **54**, 1515–1518 (2005).
51. Schroeder, K. W., Tremaine, W. J. & Ilstrup, D. M. Coated Oral 5-Aminosalicylic Acid Therapy for Mildly to Moderately Active Ulcerative Colitis. *N. Engl. J. Med.* **317**, 1625–1629 (1987).
52. Naegeli, A. N. *et al.* Full, Partial, and Modified Permutations of the Mayo Score: Characterizing Clinical and Patient-Reported Outcomes in Ulcerative Colitis Patients. *Crohns Colitis 360* **3**, otab007 (2021).
53. Peyrin-Biroulet, L. Clinical Remission As Defined by the Mayo Score: Do We Deceive Ourselves? *Inflamm. Bowel Dis.* **21**, E2–E3 (2015).
54. Lewis, J. D. *et al.* Use of the noninvasive components of the mayo score to assess clinical response in Ulcerative Colitis: *Inflamm. Bowel Dis.* **14**, 1660–1666 (2008).
55. Linares, R., Francés, R., Gutiérrez, A. & Juanola, O. Bacterial Translocation as Inflammatory Driver in Crohn's Disease. *Front. Cell Dev. Biol.* **9**, 703310 (2021).
56. Antoni, L. Intestinal barrier in inflammatory bowel disease. *World J. Gastroenterol.* **20**, 1165 (2014).
57. Gersemann, M. *et al.* Differences in goblet cell differentiation between Crohn's disease and ulcerative colitis. *Differentiation* **77**, 84–94 (2009).
58. Lazaridis, L.-D. *et al.* Activation of NLRP3 Inflammasome in Inflammatory Bowel Disease: Differences Between Crohn's Disease and Ulcerative Colitis. *Dig. Dis. Sci.* **62**, 2348–2356 (2017).
59. Ranson, N. *et al.* Nod-Like Receptor Pyrin-Containing Protein 6 (NLRP6) Is Up-regulated in Ileal Crohn's Disease and Differentially Expressed in Goblet Cells. *Cell. Mol. Gastroenterol. Hepatol.* **6**, 110-112.e8 (2018).
60. Gao, P. *et al.* The Crohn Disease-associated ATG16L1 ^{T300A} polymorphism regulates inflammatory responses by modulating TLR- and NLR-mediated signaling. *Autophagy* **18**, 2561–2575 (2022).

61. Jakubzick, C. V., Randolph, G. J. & Henson, P. M. Monocyte differentiation and antigen-presenting functions. *Nat. Rev. Immunol.* **17**, 349–362 (2017).
62. Hopkinson-Woolley, J., Hughes, D., Gordon, S. & Martin, P. Macrophage recruitment during limb development and wound healing in the embryonic and foetal mouse. *J. Cell Sci.* **107**, 1159–1167 (1994).
63. Hashimoto, D. *et al.* Tissue-Resident Macrophages Self-Maintain Locally throughout Adult Life with Minimal Contribution from Circulating Monocytes. *Immunity* **38**, 792–804 (2013).
64. Hume, D. A. The mononuclear phagocyte system. *Curr. Opin. Immunol.* **18**, 49–53 (2006).
65. Caër, C. & Wick, M. J. Human Intestinal Mononuclear Phagocytes in Health and Inflammatory Bowel Disease. *Front. Immunol.* **11**, (2020).
66. Pei, Y. & Yeo, Y. Drug delivery to macrophages: Challenges and opportunities. *J. Controlled Release* **240**, 202–211 (2016).
67. Ożańska, A., Szymczak, D. & Rybka, J. Pattern of human monocyte subpopulations in health and disease. *Scand. J. Immunol.* **92**, (2020).
68. Anderson, A. *et al.* Monocytosis Is a Biomarker of Severity in Inflammatory Bowel Disease: Analysis of a 6-Year Prospective Natural History Registry. *Inflamm. Bowel Dis.* **28**, 70–78 (2022).
69. Li, D. *et al.* Inflammation in the peripheral blood system of Crohn's Disease. *Clin. Exp. Med.* (2023) doi:10.1007/s10238-023-01030-3.
70. Ushach, I. & Zlotnik, A. Biological role of granulocyte macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) on cells of the myeloid lineage. *J. Leukoc. Biol.* **100**, 481–489 (2016).
71. Sun, T., Nguyen, A. & Gommerman, J. L. Dendritic Cell Subsets in Intestinal Immunity and Inflammation. *J. Immunol.* **204**, 1075–1083 (2020).
72. Stagg, A. J. Intestinal Dendritic Cells in Health and Gut Inflammation. *Front. Immunol.* **9**, 2883 (2018).
73. Rescigno, M. *et al.* Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nat. Immunol.* **2**, 361–367 (2001).
74. Magnusson, M. K. & Wick, M. J. Intestinal dendritic cell and macrophage subsets: Tipping the balance to Crohn's Disease? *Eur. J. Microbiol. Immunol.* **1**, 19–24 (2011).
75. Magnusson, M. K. *et al.* Macrophage and dendritic cell subsets in IBD: ALDH+ cells are reduced in colon tissue of patients with ulcerative colitis regardless of inflammation. *Mucosal Immunol.* **9**, 171–182 (2016).
76. Faleiro, R. *et al.* Crohn's disease is facilitated by a disturbance of programmed death-1 ligand 2 on blood dendritic cells. *Clin. Transl. Immunol.* **8**, (2019).

77. El Sayed, S. *et al.* CCR2 promotes monocyte recruitment and intestinal inflammation in mice lacking the interleukin-10 receptor. *Sci. Rep.* **12**, 452 (2022).
78. Bain, C. C. *et al.* Constant replenishment from circulating monocytes maintains the macrophage pool in the intestine of adult mice. *Nat. Immunol.* **15**, 929–937 (2014).
79. Bujko, A. *et al.* Transcriptional and functional profiling defines human small intestinal macrophage subsets. *J. Exp. Med.* **215**, 441–458 (2018).
80. Beitnes, A.-C. R. *et al.* Rapid Accumulation of CD14+CD11c+ Dendritic Cells in Gut Mucosa of Celiac Disease after in vivo Gluten Challenge. *PLoS ONE* **7**, e33556 (2012).
81. Thiesen, S. *et al.* CD14hiHLA-DRdim macrophages, with a resemblance to classical blood monocytes, dominate inflamed mucosa in Crohn's disease. *J. Leukoc. Biol.* **95**, 531–541 (2013).
82. Na, Y. R., Stakenborg, M., Seok, S. H. & Matteoli, G. Macrophages in intestinal inflammation and resolution: a potential therapeutic target in IBD. *Nat. Rev. Gastroenterol. Hepatol.* **16**, 531–543 (2019).
83. Artis, D. Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut. *Nat Rev Immunol* **8**, (2008).
84. Gabanyi, I. *et al.* Neuro-immune Interactions Drive Tissue Programming in Intestinal Macrophages. *Cell* **164**, 378–391 (2016).
85. Chiaranunt, P., Tai, S. L., Ngai, L. & Mortha, A. Beyond Immunity: Underappreciated Functions of Intestinal Macrophages. *Front. Immunol.* **12**, 749708 (2021).
86. Grainger, J. R. *et al.* Inflammatory monocytes regulate pathologic responses to commensals during acute gastrointestinal infection. *Nat. Med.* **19**, 713–721 (2013).
87. A-Gonzalez, N. *et al.* Phagocytosis imprints heterogeneity in tissue-resident macrophages. *J. Exp. Med.* **214**, 1281–1296 (2017).
88. Kamada, N. *et al.* Human CD14+ Macrophages in Intestinal Lamina Propria Exhibit Potent Antigen-Presenting Ability. *J. Immunol.* **183**, 1724–1731 (2009).
89. Meriwether, D. *et al.* Macrophage COX2 Mediates Efferocytosis, Resolution Reprogramming, and Intestinal Epithelial Repair. *Cell. Mol. Gastroenterol. Hepatol.* **13**, 1095–1120 (2022).
90. Liebold, I., Jawazneh, A. A., Hamley, M. & Bosurgi, L. Apoptotic cell signals and heterogeneity in macrophage function: Fine-tuning for a healthy liver. *Semin. Cell Dev. Biol.* **119**, 72–81 (2021).
91. Vos, A. C. W. *et al.* Regulatory macrophages induced by infliximab are involved in healing in vivo and in vitro: *Inflamm. Bowel Dis.* **18**, 401–408 (2012).
92. Lissner, D. *et al.* Monocyte and M1 Macrophage-induced Barrier Defect Contributes to Chronic Intestinal Inflammation in IBD: *Inflamm. Bowel Dis.* 1 (2015) doi:10.1097/MIB.0000000000000384.

93. Kamada, N. *et al.* Unique CD14+ intestinal macrophages contribute to the pathogenesis of Crohn disease via IL-23/IFN-γ axis. *J. Clin. Invest.* JCI34610 (2008) doi:10.1172/JCI34610.
94. Rugtveit, J. *et al.* Cytokine profiles differ in newly recruited and resident subsets of mucosal macrophages from inflammatory bowel disease. *Gastroenterology* **112**, 1493–1505 (1997).
95. Abbas, A. K., Lichtman, A. H. & Pillai, S. *Basic immunology: functions and disorders of the immune system*. (Elsevier, 2020).
96. Patel, A. A. *et al.* The fate and lifespan of human monocyte subsets in steady state and systemic inflammation. *J. Exp. Med.* **214**, 1913–1923 (2017).
97. Grimm, M. *et al.* Direct evidence of monocyte recruitment to inflammatory bowel disease mucosa. *J. Gastroenterol. Hepatol.* **10**, 387–395 (1995).
98. Gautier, E. L., Ivanov, S., Lesnik, P. & Randolph, G. J. Local apoptosis mediates clearance of macrophages from resolving inflammation in mice. *Blood* **122**, 2714–2722 (2013).
99. Janssen, W. J. *et al.* Fas Determines Differential Fates of Resident and Recruited Macrophages during Resolution of Acute Lung Injury. *Am. J. Respir. Crit. Care Med.* **184**, 547–560 (2011).
100. Denning, T. L. *et al.* Functional Specializations of Intestinal Dendritic Cell and Macrophage Subsets That Control Th17 and Regulatory T Cell Responses Are Dependent on the T Cell/APC Ratio, Source of Mouse Strain, and Regional Localization. *J. Immunol.* **187**, 733–747 (2011).
101. Domanska, D. *et al.* Single-cell transcriptomic analysis of human colonic macrophages reveals niche-specific subsets. *J. Exp. Med.* **219**, e20211846 (2022).
102. Matheis, F. *et al.* Adrenergic Signaling in Muscularis Macrophages Limits Infection-Induced Neuronal Loss. *Cell* **180**, 64-78.e16 (2020).
103. De Schepper, S. *et al.* Self-Maintaining Gut Macrophages Are Essential for Intestinal Homeostasis. *Cell* **175**, 400-415.e13 (2018).
104. Martin, J. C. *et al.* Single-Cell Analysis of Crohn's Disease Lesions Identifies a Pathogenic Cellular Module Associated with Resistance to Anti-TNF Therapy. *Cell* **178**, 1493-1508.e20 (2019).
105. Bernardo, D. *et al.* Human intestinal pro-inflammatory CD11chighCCR2+CX3CR1+ macrophages, but not their tolerogenic CD11c−CCR2−CX3CR1− counterparts, are expanded in inflammatory bowel disease. *Mucosal Immunol.* **11**, 1114–1126 (2018).
106. Matusiak, M. *et al. A spatial map of human macrophage niches links tissue location with function*. http://biorxiv.org/lookup/doi/10.1101/2022.08.18.504434 (2022) doi:10.1101/2022.08.18.504434.

107. Mao, L. *et al.* Loss-of-function CARD8 mutation causes NLRP3 inflammasome activation and Crohn's disease. *J. Clin. Invest.* **128**, 1793–1806 (2018).
108. Wright, P. B. *et al.* The mannose receptor (CD206) identifies a population of colonic macrophages in health and inflammatory bowel disease. *Sci. Rep.* **11**, 19616 (2021).
109. Van Der Meer, J. H. M. *et al.* Anatomical Variation in Mesenteric Macrophage Phenotypes in Crohn's Disease. *Clin. Transl. Gastroenterol.* **11**, e00198 (2020).
110. Gwak, S.-Y. *et al.* Potential Role of Heme Oxygenase-1 in the Resolution of Experimentally Induced Colitis through Regulation of Macrophage Polarization. *Gut Liver* **16**, 246–258 (2022).
111. Maiguel, D. *et al.* Small Molecule–Mediated Activation of the Integrin CD11b/CD18 Reduces Inflammatory Disease. *Sci. Signal.* **4**, (2011).
112. Bermudez, H. *et al.* P142 CONSTITUTIVELY ACTIVE MAC-1 (CD11B) AMELIORATES INFLAMMATION IN MOUSE DSS COLITIS MODEL. *Gastroenterology* **156**, S95 (2019).
113. Schif-Zuck, S. *et al.* Saturated-efferocytosis generates pro-resolving CD11blow macrophages: Modulation by resolvins and glucocorticoids. *Eur. J. Immunol.* **41**, 366–379 (2011).
114. Greenberg, M. E. *et al.* Oxidized phosphatidylserine–CD36 interactions play an essential role in macrophage-dependent phagocytosis of apoptotic cells. *J. Exp. Med.* **203**, 2613–2625 (2006).
115. Pennathur, S. *et al.* The Macrophage Phagocytic Receptor CD36 Promotes Fibrogenic Pathways on Removal of Apoptotic Cells during Chronic Kidney Injury. *Am. J. Pathol.* **185**, 2232–2245 (2015).
116. Park, H.-T. *et al.* Revealing immune responses in the Mycobacterium avium subsp. paratuberculosis-infected THP-1 cells using single cell RNA-sequencing. *PLOS ONE* **16**, e0254194 (2021).
117. Zhao, L. *et al.* CD36 Senses Dietary Lipids and Regulates Lipids Homeostasis in the Intestine. *Front. Physiol.* **12**, 669279 (2021).
118. Xiong, W., Frasch, S. C., Thomas, S. M., Bratton, D. L. & Henson, P. M. Induction of TGF-β1 Synthesis by Macrophages in Response to Apoptotic Cells Requires Activation of the Scavenger Receptor CD36. *PLoS ONE* **8**, e72772 (2013).
119. Dige, A. *et al.* Soluble CD163, a Specific Macrophage Activation Marker, is Decreased by Anti-TNF- *α* Antibody Treatment in Active Inflammatory Bowel Disease. *Scand. J. Immunol.* **80**, 417–423 (2014).
120. Franzè, E. *et al.* Lesional Accumulation of CD163-Expressing Cells in the Gut of Patients with Inflammatory Bowel Disease. *PLoS ONE* **8**, e69839 (2013).
121. Barros, M. H. M., Hauck, F., Dreyer, J. H., Kempkes, B. & Niedobitek, G. Macrophage Polarisation: an Immunohistochemical

Approach for Identifying M1 and M2 Macrophages. *PLoS ONE* **8**, e80908 (2013).
122. Rogler, G. *et al.* T-cell co-stimulatory molecules are upregulated on intestinal macrophages from inflammatory bowel disease mucosa: *Eur. J. Gastroenterol. Hepatol.* **11**, 1105–1112 (1999).
123. Liu, Z. X., Hiwatashi, N., Noguchi, M. & Toyota, T. Increased Expression of Costimulatory Molecules on Peripheral Blood Monocytes in Patients with Crohn's Disease. *Scand. J. Gastroenterol.* **32**, 1241–1246 (1997).
124. Tillinger, W. *et al.* Expression of the High-Affinity IgG Receptor FcRI (CD64) in Patients With Inflammatory Bowel Disease: A New Biomarker for Gastroenterologic Diagnostics. *Am. J. Gastroenterol.* **104**, 102–109 (2009).
125. Minar, P. *et al.* Utility of Neutrophil Fcγ Receptor I (CD64) Index as a Biomarker for Mucosal Inflammation in Pediatric Crohn's Disease: *Inflamm. Bowel Dis.* 1 (2014) doi:10.1097/MIB.0000000000000049.
126. Minar, P. *et al.* Validation of Neutrophil CD64 Blood Biomarkers to Detect Mucosal Inflammation in Pediatric Crohn's Disease. *Inflamm. Bowel Dis.* **24**, 198–208 (2018).
127. Tamoutounour, S. *et al.* CD64 distinguishes macrophages from dendritic cells in the gut and reveals the Th1-inducing role of mesenteric lymph node macrophages during colitis: HIGHLIGHTS. *Eur. J. Immunol.* **42**, 3150–3166 (2012).
128. Bruhns, P. *et al.* Specificity and affinity of human Fcγ receptors and their polymorphic variants for human IgG subclasses. *Blood* **113**, 3716–3725 (2009).
129. Chen, Q., Nair, S. & Ruedl, C. Microbiota regulates the turnover kinetics of gut macrophages in health and inflammation. *Life Sci. Alliance* **5**, e202101178 (2022).
130. Chiaranunt, P. *et al.* Microbial energy metabolism fuels an intestinal macrophage niche in solitary isolated lymphoid tissues through purinergic signaling. *Sci. Immunol.* **8**, eabq4573 (2023).
131. Franchi, L., Muñoz-Planillo, R. & Núñez, G. Sensing and reacting to microbes through the inflammasomes. *Nat. Immunol.* **13**, 325–332 (2012).
132. Shaw, M. H., Kamada, N., Kim, Y.-G. & Núñez, G. Microbiota-induced IL-1β, but not IL-6, is critical for the development of steady-state TH17 cells in the intestine. *J. Exp. Med.* **209**, 251–258 (2012).
133. Chen, L. *et al.* The role of Th17 cells in inflammatory bowel disease and the research progress. *Front. Immunol.* **13**, 1055914 (2023).
134. Kim, C. H. Immune regulation by microbiome metabolites. *Immunology* **154**, 220–229 (2018).

135. Galazzo, G. *et al.* Faecal Microbiota Dynamics and their Relation to Disease Course in Crohn's Disease. *J. Crohns Colitis* **13**, 1273–1282 (2019).
136. Strömbeck, A. *et al.* Fecal microbiota composition is linked to the postoperative disease course in patients with Crohn's disease. *BMC Gastroenterol.* **20**, 130 (2020).
137. Gallagher, K., Catesson, A., Griffin, J. L., Holmes, E. & Williams, H. R. T. Metabolomic Analysis in Inflammatory Bowel Disease: A Systematic Review. *J. Crohns Colitis* jjaa227 (2020) doi:10.1093/ecco-jcc/jjaa227.
138. Meelu, P. *et al.* Impaired Innate Immune Function Associated with Fecal Supernatant from Crohn's Disease Patients: Insights into Potential Pathogenic Role of the Microbiome. *Inflamm. Bowel Dis.* **20**, 1139–1146 (2014).
139. Maasfeh, L. *et al.* Impaired Luminal Control of Intestinal Macrophage Maturation in Patients With Ulcerative Colitis During Remission. *Cell. Mol. Gastroenterol. Hepatol.* **12**, 1415–1432 (2021).
140. Yang, C., Long, D., Sung, J., Alghoul, Z. & Merlin, D. Orally Administered Natural Lipid Nanoparticle-Loaded 6-Shogaol Shapes the Anti-Inflammatory Microbiota and Metabolome. *Pharmaceutics* **13**, 1355 (2021).
141. Chapuy, L. *et al.* Two distinct colonic CD14+ subsets characterized by single-cell RNA profiling in Crohn's disease. *Mucosal Immunol.* **12**, 703–719 (2019).
142. Albers, R. *et al.* Markers to measure immunomodulation in human nutrition intervention studies. *Br. J. Nutr.* **94**, 452–481 (2005).
143. Gordon, S. & Martinez, F. O. Alternative Activation of Macrophages: Mechanism and Functions. *Immunity* **32**, 593–604 (2010).
144. Zhou, G. *et al.* CD177 $^{+}$ neutrophils as functionally activated neutrophils negatively regulate IBD. *Gut* **67**, 1052–1063 (2018).
145. Yao, Z. *et al.* Neutrophil Infiltration Characterized by Upregulation of S100A8, S100A9, S100A12 and CXCR2 Is Associated With the Co-Occurrence of Crohn's Disease and Peripheral Artery Disease. *Front. Immunol.* **13**, 896645 (2022).
146. Brynjolfsson, S. F. *et al.* An Antibody Against Triggering Receptor Expressed on Myeloid Cells 1 (TREM-1) Dampens Proinflammatory Cytokine Secretion by Lamina Propria Cells from Patients with IBD: *Inflamm. Bowel Dis.* **22**, 1803–1811 (2016).
147. Therrien, A. *et al.* Recruitment of activated neutrophils correlates with disease severity in adult Crohn's disease. *Clin. Exp. Immunol.* **195**, 251–264 (2019).
148. Summers, C. *et al.* Neutrophil kinetics in health and disease. *Trends Immunol.* **31**, 318–324 (2010).
149. Hidalgo, A., Chilvers, E. R., Summers, C. & Koenderman, L. The Neutrophil Life Cycle. *Trends Immunol.* **40**, 584–597 (2019).

150. Laan, M. *et al.* A role of GM-CSF in the accumulation of neutrophils in the airways caused by IL-17 and TNF-α. *Eur. Respir. J.* **21**, 387–393 (2003).
151. Murphy, K. & Weaver, C. *Janeway's immunobiology*. (Garland Science/Taylor & Francis Group, LLC, 2016).
152. Saez, A., Herrero-Fernandez, B., Gomez-Bris, R., Sánchez-Martinez, H. & Gonzalez-Granado, J. M. Pathophysiology of Inflammatory Bowel Disease: Innate Immune System. *Int. J. Mol. Sci.* **24**, 1526 (2023).
153. Wéra, O., Lancellotti, P. & Oury, C. The Dual Role of Neutrophils in Inflammatory Bowel Diseases. *J. Clin. Med.* **5**, 118 (2016).
154. Li, T. *et al.* Neutrophil Extracellular Traps Induce Intestinal Damage and Thrombotic Tendency in Inflammatory Bowel Disease. *J. Crohns Colitis* **14**, 240–253 (2020).
155. Lin, E. Y.-H. *et al.* Neutrophil Extracellular Traps Impair Intestinal Barrier Function during Experimental Colitis. *Biomedicines* **8**, 275 (2020).
156. Higuchi, Y. *et al.* Gastrointestinal Fibroblasts Have Specialized, Diverse Transcriptional Phenotypes: A Comprehensive Gene Expression Analysis of Human Fibroblasts. *PLOS ONE* **10**, e0129241 (2015).
157. Pandey, A., Kurera, M. & Man, S. M. Primary Intestinal Fibroblasts: Isolation, Cultivation, and Maintenance. in *Inflammation and Cancer* (ed. Jenkins, B. J.) vol. 2691 327–335 (Springer US, 2023).
158. Gomes, R. N., Manuel, F. & Nascimento, D. S. The bright side of fibroblasts: molecular signature and regenerative cues in major organs. *Npj Regen. Med.* **6**, 43 (2021).
159. Bautista-Hernández, L. A., Gómez-Olivares, J. L., Buentello-Volante, B. & Bautista-de Lucio, V. M. Fibroblasts: the unknown sentinels eliciting immune responses against microorganisms. *Eur. J. Microbiol. Immunol.* **7**, 151–157 (2017).
160. Kim, Y.-G. *et al.* The Nod2 Sensor Promotes Intestinal Pathogen Eradication via the Chemokine CCL2-Dependent Recruitment of Inflammatory Monocytes. *Immunity* **34**, 769–780 (2011).
161. Ke, B.-J. *et al. Intercellular interaction between FAP fibroblasts and CD150 inflammatory monocytes mediates fibro-stenosis in Crohn's disease*. http://biorxiv.org/lookup/doi/10.1101/2023.05.04.539383 (2023) doi:10.1101/2023.05.04.539383.
162. Hayashi, Y. & Nakase, H. The Molecular Mechanisms of Intestinal Inflammation and Fibrosis in Crohn's Disease. *Front. Physiol.* **13**, 845078 (2022).
163. Lawrance, I. C., Maxwell, L. & Doe, W. Inflammation Location, But Not Type, Determines the Increase in TGF-β1 and IGF-1 Expression and Collagen Deposition in IBD Intestine: *Inflamm. Bowel Dis.* **7**, 16–26 (2001).

164. Scharl, M. *et al.* Hallmarks of epithelial to mesenchymal transition are detectable in Crohn's disease associated intestinal fibrosis. *Clin. Transl. Med.* **4**, (2015).
165. Truffi, M. *et al.* Inhibition of Fibroblast Activation Protein Restores a Balanced Extracellular Matrix and Reduces Fibrosis in Crohn's Disease Strictures Ex Vivo. *Inflamm. Bowel Dis.* **24**, 332–345 (2018).
166. Fukata, M. & Arditi, M. The role of pattern recognition receptors in intestinal inflammation. *Mucosal Immunol.* **6**, 451–463 (2013).
167. Li, D. & Wu, M. Pattern recognition receptors in health and diseases. *Signal Transduct. Target. Ther.* **6**, 291 (2021).
168. Martinon, F., Burns, K. & Tschopp, J. The Inflammasome. *Mol. Cell* **10**, 417–426 (2002).
169. Kelley, N., Jeltema, D., Duan, Y. & He, Y. The NLRP3 Inflammasome: An Overview of Mechanisms of Activation and Regulation. *Int. J. Mol. Sci.* **20**, 3328 (2019).
170. Kim, J.-H. *et al.* NLRP3 Inflammasome Activation in THP-1 Target Cells Triggered by Pathogenic Naegleria fowleri. *Infect. Immun.* **84**, 2422–2428 (2016).
171. Busch, M., Ramachandran, H., Wahle, T., Rossi, A. & Schins, R. P. F. Investigating the Role of the NLRP3 Inflammasome Pathway in Acute Intestinal Inflammation: Use of THP-1 Knockout Cell Lines in an Advanced Triple Culture Model. *Front. Immunol.* **13**, 898039 (2022).
172. Massaccesi, G. & Chattergoon, M. A. Assessment of inflammasome and type I IFN responses to DNA viruses and DNA PAMPS. in *Methods in Enzymology* vol. 625 269–285 (Elsevier, 2019).
173. Man, S. M. Inflammasomes in the gastrointestinal tract: infection, cancer and gut microbiota homeostasis. *Nat. Rev. Gastroenterol. Hepatol.* **15**, 721–737 (2018).
174. Guermonprez, P. & Helft, J. Inflammasome activation: a monocyte lineage privilege. *Nat. Immunol.* **20**, 383–385 (2019).
175. Ghimire, L., Paudel, S., Jin, L. & Jeyaseelan, S. The NLRP6 inflammasome in health and disease. *Mucosal Immunol.* **13**, 388–398 (2020).
176. Lei-Leston, A. C., Murphy, A. G. & Maloy, K. J. Epithelial Cell Inflammasomes in Intestinal Immunity and Inflammation. *Front. Immunol.* **8**, (2017).
177. Pandey, A., Shen, C., Feng, S. & Man, S. M. Cell biology of inflammasome activation. *Trends Cell Biol.* **31**, 924–939 (2021).
178. Pellegrini, C., Antonioli, L., Lopez-Castejon, G., Blandizzi, C. & Fornai, M. Canonical and Non-Canonical Activation of NLRP3 Inflammasome at the Crossroad between Immune Tolerance and Intestinal Inflammation. *Front. Immunol.* **8**, (2017).
179. Chan, A. H. & Schroder, K. Inflammasome signaling and regulation of interleukin-1 family cytokines. *J. Exp. Med.* **217**, (2020).

180. Liu, X. *et al.* Inflammasome-activated gasdermin D causes pyroptosis by forming membrane pores. *Nature* **535**, 153–158 (2016).
181. Franchi, L. *et al.* NLRC4-driven production of IL-1β discriminates between pathogenic and commensal bacteria and promotes host intestinal defense. *Nat. Immunol.* **13**, 449–456 (2012).
182. Seo, S.-U. *et al.* Distinct Commensals Induce Interleukin-1β via NLRP3 Inflammasome in Inflammatory Monocytes to Promote Intestinal Inflammation in Response to Injury. *Immunity* **42**, 744–755 (2015).
183. Otani, K. *et al.* Colchicine prevents NSAID-induced small intestinal injury by inhibiting activation of the NLRP3 inflammasome. *Sci. Rep.* **6**, 32587 (2016).
184. Seregin, S. S. *et al.* NLRP6 function in inflammatory monocytes reduces susceptibility to chemically induced intestinal injury. *Mucosal Immunol.* **10**, 434–445 (2017).
185. Seregin, S. S. *et al.* NLRP6 Protects Il10 Mice from Colitis by Limiting Colonization of Akkermansia muciniphila. *Cell Rep.* **19**, 733–745 (2017).
186. Seshadri, S., Duncan, M. D., Hart, J. M., Gavrilin, M. A. & Wewers, M. D. Pyrin Levels in Human Monocytes and Monocyte-Derived Macrophages Regulate IL-1β Processing and Release. *J. Immunol.* **179**, 1274–1281 (2007).
187. Ozato, K., Shin, D.-M., Chang, T.-H. & Morse, H. C. TRIM family proteins and their emerging roles in innate immunity. *Nat. Rev. Immunol.* **8**, 849–860 (2008).
188. Rajsbaum, R., Stoye, J. P. & O'Garra, A. Type I interferon-dependent and -independent expression of tripartite motif proteins in immune cells. *Eur. J. Immunol.* **38**, 619–630 (2008).
189. Papadopoulos, V. P., Antoniadou, C., Ritis, K. & Skendros, P. MEFV Mutations in IBD Patients: A Systematic Review and Meta-analysis. *J. Gastrointestin. Liver Dis.* **31**, 85–97 (2022).
190. Park, Y. H., Wood, G., Kastner, D. L. & Chae, J. J. Pyrin inflammasome activation and RhoA signaling in the autoinflammatory diseases FMF and HIDS. *Nat. Immunol.* **17**, 914–921 (2016).
191. Xu, H. *et al.* Innate immune sensing of bacterial modifications of Rho GTPases by the Pyrin inflammasome. *Nature* **513**, 237–241 (2014).
192. Siskind, S., Brenner, M. & Wang, P. TREM-1 Modulation Strategies for Sepsis. *Front. Immunol.* **13**, 907387 (2022).
193. Biagioli, M. *et al.* Genetic and Pharmacological Dissection of the Role of Spleen Tyrosine Kinase (Syk) in Intestinal Inflammation and Immune Dysfunction in Inflammatory Bowel Diseases. *Inflamm. Bowel Dis.* **24**, 123–135 (2018).
194. Carrasco, K. *et al.* TREM-1 multimerization is essential for its activation on monocytes and neutrophils. *Cell. Mol. Immunol.* **16**, 460–472 (2019).

195. Bouchon, A., Dietrich, J. & Colonna, M. Cutting Edge: Inflammatory Responses Can Be Triggered by TREM-1, a Novel Receptor Expressed on Neutrophils and Monocytes. *J. Immunol.* **164**, 4991–4995 (2000).
196. Tammaro, A. *et al.* TREM-1 and its potential ligands in non-infectious diseases: from biology to clinical perspectives. *Pharmacol. Ther.* **177**, 81–95 (2017).
197. Read, C. B. *et al.* Cutting Edge: Identification of Neutrophil PGLYRP1 as a Ligand for TREM-1. *J. Immunol.* **194**, 1417–1421 (2015).
198. Nguyen-Lefebvre, A. T. *et al.* The innate immune receptor TREM-1 promotes liver injury and fibrosis. *J. Clin. Invest.* **128**, 4870–4883 (2018).
199. Schenk, M., Bouchon, A., Seibold, F. & Mueller, C. TREM-1–expressing intestinal macrophages crucially amplify chronic inflammation in experimental colitis and inflammatory bowel diseases. *J. Clin. Invest.* **117**, 3097–3106 (2007).
200. Bleharski, J. R. *et al.* A Role for Triggering Receptor Expressed on Myeloid Cells-1 in Host Defense During the Early-Induced and Adaptive Phases of the Immune Response. *J. Immunol.* **170**, 3812–3818 (2003).
201. Weber, B. *et al.* TREM-1 Deficiency Can Attenuate Disease Severity without Affecting Pathogen Clearance. *PLoS Pathog.* **10**, e1003900 (2014).
202. Kökten, T. *et al.* TREM-1 Inhibition Restores Impaired Autophagy Activity and Reduces Colitis in Mice. *J. Crohns Colitis* **12**, 230–244 (2018).
203. Gaujoux, R. *et al.* Cell-centred meta-analysis reveals baseline predictors of anti-TNFα non-response in biopsy and blood of patients with IBD. *Gut* **68**, 604–614 (2019).
204. Verstockt, B. *et al.* TREM-1, the ideal predictive biomarker for endoscopic healing in anti-TNF-treated Crohn's disease patients? *Gut* **68**, 1531–1533 (2019).
205. Bendix, M. *et al.* Decrease in Mucosal IL17A, IFNγ and IL10 Expressions in Active Crohn's Disease Patients Treated with High-Dose Vitamin D Alone or Combined with Infliximab. *Nutrients* **12**, 3699 (2020).
206. Bak, N. F. *et al.* High-dose vitamin D3 supplementation decreases the number of colonic CD103+ dendritic cells in healthy subjects. *Eur. J. Nutr.* **57**, 2607–2619 (2018).
207. Riza Koksal, A. *et al.* How does a biopsy of endoscopically normal terminal ileum contribute to the diagnosis? Which patients should undergo biopsy? *Libyan J. Med.* **9**, 23441 (2014).
208. Wyczalkowska-Tomasik, A., Czarkowska-Paczek, B., Zielenkiewicz, M. & Paczek, L. Inflammatory Markers Change with Age,

but do not Fall Beyond Reported Normal Ranges. *Arch. Immunol. Ther. Exp. (Warsz.)* **64**, 249–254 (2016).
209. Mayer, A. T. *et al.* A tissue atlas of ulcerative colitis revealing evidence of sex-dependent differences in disease-driving inflammatory cell types and resistance to TNF inhibitor therapy. *Sci. Adv.* **9**, eadd1166 (2023).
210. Lu, Y., Ahmed, S., Harari, F. & Vahter, M. Impact of Ficoll density gradient centrifugation on major and trace element concentrations in erythrocytes and blood plasma. *J. Trace Elem. Med. Biol.* **29**, 249–254 (2015).
211. Fawkner-Corbett, D., Gerós, A. S., Antanaviciute, A. & Simmons, A. Isolation of human fetal intestinal cells for single-cell RNA sequencing. *STAR Protoc.* **2**, 100890 (2021).
212. Gracz, A. D., Puthoff, B. J. & Magness, S. T. Identification, Isolation, and Culture of Intestinal Epithelial Stem Cells from Murine Intestine. in *Somatic Stem Cells* (ed. Singh, S. R.) vol. 879 89–107 (Humana Press, 2012).
213. Pan, D., Das, A., Liu, D., Veazey, R. S. & Pahar, B. Isolation and Characterization of Intestinal Epithelial Cells from Normal and SIV-Infected Rhesus Macaques. *PLoS ONE* **7**, e30247 (2012).
214. Doyle, C. M. *et al.* Optimal Isolation Protocols for Examining and Interrogating Mononuclear Phagocytes From Human Intestinal Tissue. *Front. Immunol.* **12**, 727952 (2021).
215. Uniken Venema, W. T. C. *et al.* Gut mucosa dissociation protocols influence cell type proportions and single-cell gene expression levels. *Sci. Rep.* **12**, 9897 (2022).
216. Han, X., Ding, S., Jiang, H. & Liu, G. Roles of Macrophages in the Development and Treatment of Gut Inflammation. *Front. Cell Dev. Biol.* **9**, 625423 (2021).
217. Bain, C. C. & Mowat, A. McI. Macrophages in intestinal homeostasis and inflammation. *Immunol. Rev.* **260**, 102–117 (2014).
218. Prins, M. M. *et al.* Monocyte TREM-1 Levels Associate With Anti-TNF Responsiveness in IBD Through Autophagy and Fcγ-Receptor Signaling Pathways. *Front. Immunol.* **12**, 627535 (2021).
219. NanoString, nCounter Technology. (2023).
220. Kourtzelis, I., Hajishengallis, G. & Chavakis, T. Phagocytosis of Apoptotic Cells in Resolution of Inflammation. *Front. Immunol.* **11**, 553 (2020).
221. Schmidt, T. *et al.* CD66b overexpression and homotypic aggregation of human peripheral blood neutrophils after activation by a gram-positive stimulus. *J. Leukoc. Biol.* **91**, 791–802 (2012).
222. Erriah, M. *et al.* Galectin-3 enhances monocyte-derived macrophage efferocytosis of apoptotic granulocytes in asthma. *Respir. Res.* **20**, 1 (2019).

223. Lin, J. *et al.* MerTK-mediated efferocytosis promotes immune tolerance and tumor progression in osteosarcoma through enhancing M2 polarization and PD-L1 expression. *OncoImmunology* **11**, 2024941 (2022).
224. Evans, A. L., Blackburn, J. W. D., Yin, C. & Heit, B. Quantitative Efferocytosis Assays. in *Phagocytosis and Phagosomes* (ed. Botelho, R.) vol. 1519 25–41 (Springer New York, 2017).
225. Zaidan, I. *et al.* Angiotensin-(1-7)/MasR axis promotes migration of monocytes/macrophages with a regulatory phenotype to perform phagocytosis and efferocytosis. *JCI Insight* **7**, e147819 (2022).
226. Snodgrass, R. G. *et al.* Efferocytosis potentiates the expression of arachidonate 15-lipoxygenase (ALOX15) in alternatively activated human macrophages through LXR activation. *Cell Death Differ.* **28**, 1301–1316 (2021).
227. Lee, J. *et al.* Alpha 1 Antitrypsin-Deficient Macrophages Have Impaired Efferocytosis of Apoptotic Neutrophils. *Front. Immunol.* **11**, 574410 (2020).
228. Salina, A. C. G. *et al. Efferocytosis of SARS-CoV-2-infected dying cells impairs macrophage anti-inflammatory functions and clearance of apoptotic cells*. http://medrxiv.org/lookup/doi/10.1101/2021.02.18.21251504 (2021) doi:10.1101/2021.02.18.21251504.
229. Michlewska, S., Dransfield, I., Megson, I. L. & Rossi, A. G. Macrophage phagocytosis of apoptotic neutrophils is critically regulated by the opposing actions of pro-inflammatory and anti-inflammatory agents: key role for TNF-α. *FASEB J.* **23**, 844–854 (2009).
230. Shereck, E. B., Van De Ven, C. & Cairo, M. S. Neonatal Leukocyte Physiology and Disorders. in *Avery's Diseases of the Newborn* 1108–1122 (Elsevier, 2012). doi:10.1016/B978-1-4377-0134-0.10078-2.
231. Liu, J. *et al.* Calycosin Inhibits Intestinal Fibrosis on CCD-18Co Cells via Modulating Transforming Growth Factor-β/Smad Signaling Pathway. *Pharmacology* **104**, 81–89 (2019).
232. Lewis, A. *et al.* Small-molecule Wnt inhibitors are a potential novel therapy for intestinal fibrosis in Crohns disease. *Clin. Sci.* **136**, 1405–1423 (2022).
233. Lee, S.-A. *et al.* Vitamin D attenuates myofibroblast differentiation and extracellular matrix accumulation in nasal polyp-derived fibroblasts through smad2/3 signaling pathway. *Sci. Rep.* **7**, 7299 (2017).
234. Bain, C. C. & Schridde, A. Origin, Differentiation, and Function of Intestinal Macrophages. *Front. Immunol.* **9**, 2733 (2018).
235. Savolainen, O. I., Sandberg, A.-S. & Ross, A. B. A Simultaneous Metabolic Profiling and Quantitative Multimetabolite Metabolomic Method for Human Plasma Using Gas-Chromatography Tandem Mass Spectrometry. *J. Proteome Res.* **15**, 259–265 (2016).

236. A, J. *et al.* Extraction and GC/MS Analysis of the Human Blood Plasma Metabolome. *Anal. Chem.* **77**, 8086–8094 (2005).
237. Marchesi, J. R. *et al.* The gut microbiota and host health: a new clinical frontier. *Gut* **65**, 330–339 (2016).
238. Lundberg, E. *et al.* Defining the transcriptome and proteome in three functionally different human cell lines. *Mol. Syst. Biol.* **6**, 450 (2010).
239. Vogel, C. & Marcotte, E. M. Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nat. Rev. Genet.* **13**, 227–232 (2012).
240. Bustin, S. A. *et al.* The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clin. Chem.* **55**, 611–622 (2009).
241. Bamias, G. *et al.* Comparative Study of Candidate Housekeeping Genes for Quantification of Target Gene Messenger RNA Expression by Real-Time PCR in Patients with Inflammatory Bowel Disease: *Inflamm. Bowel Dis.* **19**, 2840–2847 (2013).
242. Vandesompele, J. *et al.* Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* **3**, RESEARCH0034 (2002).
243. Hinterleitner, T. A., Saada, J. I., Berschneider, H. M., Powell, D. W. & Valentich, J. D. IL-1 stimulates intestinal myofibroblast COX gene expression and augments activation of Cl- secretion in T84 cells. *Am. J. Physiol.-Cell Physiol.* **271**, C1262–C1268 (1996).
244. Kong, X. Y. *et al.* Loss of lysosomal protein NCU-G1 results in spontaneous liver fibrosis with accumulation of lipofuscin and iron in Kupffer cells. *Dis. Model. Mech.* dmm.014050 (2014) doi:10.1242/dmm.014050.
245. Veldman-Jones, M. H. *et al.* Evaluating Robustness and Sensitivity of the NanoString Technologies nCounter Platform to Enable Multiplexed Gene Expression Analysis of Clinical Samples. *Cancer Res.* **75**, 2587–2593 (2015).
246. Moraes, L. *et al.* Systemic Inflammatory Protein Profiles Distinguish Irritable Bowel Syndrome (IBS) and Ulcerative Colitis, Irrespective of Inflammation or IBS-Like Symptoms. *Inflamm. Bowel Dis.* (2020) doi:10.1093/ibd/izz322.
247. Arango Duque, G. & Descoteaux, A. Macrophage Cytokines: Involvement in Immunity and Infectious Diseases. *Front. Immunol.* **5**, (2014).
248. Neurath, M. F. Cytokines in inflammatory bowel disease. *Nat. Rev. Immunol.* **14**, 329–342 (2014).
249. Jongsma, M. M. E. *et al.* Serum Immune Profiling in Paediatric Crohn's Disease Demonstrates Stronger Immune Modulation With First-Line Infliximab Than Conventional Therapy and Pre-Treatment Profiles

Predict Clinical Response to Both Treatments. *J. Crohns Colitis* **17**, 1262–1277 (2023).
250. Fredriksson, S. *et al.* Protein detection using proximity-dependent DNA ligation assays. *Nat. Biotechnol.* **20**, 473–477 (2002).
251. Shen, Q. *et al.* Strong impact on plasma protein profiles by precentrifugation delay but not by repeated freeze-thaw cycles, as analyzed using multiplex proximity extension assays. *Clin. Chem. Lab. Med. CCLM* **56**, 582–594 (2018).
252. Mesoscale Discovery. https://www.mesoscale.com/.
253. Wei, J. S., Badgett, T. C. & Khan, J. New technologies for diagnosing pediatric tumors. *Expert Opin. Med. Diagn.* **2**, 1205–1219 (2008).
254. Chometon, T. Q. *et al.* A protocol for rapid monocyte isolation and generation of singular human monocyte-derived dendritic cells. *PLOS ONE* **15**, e0231132 (2020).
255. Holmberg-Thyden, S., Grønbæk, K., Gang, A. O., El Fassi, D. & Hadrup, S. R. A user's guide to multicolor flow cytometry panels for comprehensive immune profiling. *Anal. Biochem.* **627**, 114210 (2021).
256. Marsman, C., Jorritsma, T., Ten Brinke, A. & Van Ham, S. M. Flow Cytometric Methods for the Detection of Intracellular Signaling Proteins and Transcription Factors Reveal Heterogeneity in Differentiating Human B Cell Subsets. *Cells* **9**, 2633 (2020).
257. Hermiston, M. L., Zikherman, J. & Zhu, J. W. CD45, CD148, and Lyp/Pep: critical phosphatases regulating Src family kinase signaling networks in immune cells. *Immunol. Rev.* **228**, 288–311 (2009).
258. Jardine, L. *et al.* Rapid Detection of Dendritic Cell and Monocyte Disorders Using CD4 as a Lineage Marker of the Human Peripheral Blood Antigen-Presenting Cell Compartment. *Front. Immunol.* **4**, (2013).
259. Huang, L. *et al.* Functions of EpCAM in physiological processes and diseases (Review). *Int. J. Mol. Med.* (2018) doi:10.3892/ijmm.2018.3764.
260. Kinchen, J. *et al.* Structural Remodeling of the Human Colonic Mesenchyme in Inflammatory Bowel Disease. *Cell* **175**, 372-386.e17 (2018).
261. Thermofisher. Amine-reactive Live/Dead Acqua dye.
262. Crowley, L. C., Marfell, B. J., Scott, A. P. & Waterhouse, N. J. Quantitation of Apoptosis and Necrosis by Annexin V Binding, Propidium Iodide Uptake, and Flow Cytometry. *Cold Spring Harb. Protoc.* **2016**, pdb.prot087288 (2016).
263. Staats, J., Divekar, A., McCoy, J. P. & Maecker, H. T. Guidelines for Gating Flow Cytometry Data for Immunological Assays. in *Immunophenotyping* (ed. McCoy, Jr, J. P.) vol. 2032 81–104 (Springer New York, 2019).

264. Miltenyi, S., Müller, W., Weichel, W. & Radbruch, A. High gradient magnetic cell separation with MACS. *Cytometry* **11**, 231–238 (1990).
265. Bhattacharjee, J., Das, B., Mishra, A., Sahay, P. & Upadhyay, P. Monocytes isolated by positive and negative magnetic sorting techniques show different molecular characteristics and immunophenotypic behaviour. *F1000Research* **6**, 2045 (2018).
266. Liao, X., Makris, M. & Luo, X. M. Fluorescence-activated Cell Sorting for Purification of Plasmacytoid Dendritic Cells from the Mouse Bone Marrow. *J. Vis. Exp.* 54641 (2016) doi:10.3791/54641.
267. Stack, E. C., Wang, C., Roman, K. A. & Hoyt, C. C. Multiplexed immunohistochemistry, imaging, and quantitation: A review, with an assessment of Tyramide signal amplification, multispectral imaging and multiplex analysis. *Methods* **70**, 46–58 (2014).
268. Immunofluorescence staining, Akoya. (2023).
269. Bobrow, M. N., Harris, T. D., Shaughnessy, K. J. & Litt, G. J. Catalyzed reporter deposition, a novel method of signal amplification application to immunoassays. *J. Immunol. Methods* **125**, 279–285 (1989).
270. Khatri, V. & Kalyanasundaram, R. Therapeutic implications of inflammasome in inflammatory bowel disease. *FASEB J.* **35**, (2021).
271. De La Fuente, M. *et al.* Escherichia coli isolates from inflammatory bowel diseases patients survive in macrophages and activate NLRP3 inflammasome. *Int. J. Med. Microbiol.* **304**, 384–392 (2014).
272. Weiser, M. *et al.* Molecular classification of Crohn's disease reveals two clinically relevant subtypes. *Gut* **67**, 36–42 (2018).
273. Boucher, G. *et al.* Serum Analyte Profiles Associated With Crohn's Disease and Disease Location. *Inflamm. Bowel Dis.* **28**, 9–20 (2022).
274. Nieto, J. C. *et al.* CSF-1 regulates the function of monocytes in Crohn's disease patients in remission. *Sci. Rep.* **7**, 92 (2017).
275. Park, J. J. *et al.* Correlation of Serum-Soluble Triggering Receptor Expressed on Myeloid Cells-1 with Clinical Disease Activity in Inflammatory Bowel Disease. *Dig. Dis. Sci.* **54**, 1525–1531 (2009).
276. Schridde, A. *et al.* Tissue-specific differentiation of colonic macrophages requires TGFβ receptor-mediated signaling. *Mucosal Immunol.* **10**, 1387–1399 (2017).
277. Gao, S. *et al.* The characteristics and pivotal roles of triggering receptor expressed on myeloid cells-1 in autoimmune diseases. *Autoimmun. Rev.* **18**, 25–35 (2019).
278. Mavropoulou, E. *et al.* Association of serum interleukin-6 and soluble interleukin-2-receptor levels with disease activity status in patients with inflammatory bowel disease: A prospective observational study. *PLOS ONE* **15**, e0233811 (2020).
279. Guo, Y. *et al.* Biological characteristics of IL-6 and related intestinal diseases. *Int. J. Biol. Sci.* **17**, 204–219 (2021).

280. Matsunaga, H. *et al.* Omega-3 polyunsaturated fatty acids ameliorate the severity of ileitis in the senescence accelerated mice (SAM)P1/Yit mice model. *Clin. Exp. Immunol.* **158**, 325–333 (2009).
281. Adegbola, S. O., Sahnan, K., Warusavitarne, J., Hart, A. & Tozer, P. Anti-TNF Therapy in Crohn's Disease. *Int. J. Mol. Sci.* **19**, 2244 (2018).
282. Bouhnik, Y. *et al.* Efficacy of adalimumab in patients with Crohn's disease and symptomatic small bowel stricture: a multicentre, prospective, observational cohort (CREOLE) study. *Gut* **67**, 53–60 (2018).
283. Gisbert, J. P., Marín, A. C., McNicholl, A. G. & Chaparro, M. Systematic review with meta-analysis: the efficacy of a second anti-TNF in patients with inflammatory bowel disease whose previous anti-TNF treatment has failed. *Aliment. Pharmacol. Ther.* **41**, 613–623 (2015).
284. Italiani, P. & Boraschi, D. From Monocytes to M1/M2 Macrophages: Phenotypical vs. Functional Differentiation. *Front. Immunol.* **5**, (2014).
285. Quiros, M. *et al.* Macrophage-derived IL-10 mediates mucosal repair by epithelial WISP-1 signaling. *J. Clin. Invest.* **127**, 3510–3520 (2017).
286. Biedroń, R., Peruń, A. & Józefowski, S. CD36 Differently Regulates Macrophage Responses to Smooth and Rough Lipopolysaccharide. *PLOS ONE* **11**, e0153558 (2016).
287. Villablanca, E. J., Selin, K. & Hedin, C. R. H. Mechanisms of mucosal healing: treating inflammatory bowel disease without immunosuppression? *Nat. Rev. Gastroenterol. Hepatol.* **19**, 493–507 (2022).
288. Loras Alastruey, C., Andújar Murcia, X. & Esteve Comas, M. The role of stents in the treatment of Crohn's disease strictures. *Endosc. Int. Open* **04**, E301–E308 (2016).
289. Yao, H. & Tang, G. Macrophages in intestinal fibrosis and regression. *Cell. Immunol.* **381**, 104614 (2022).
290. Ohue-Kitano, R. *et al.* α-Linolenic acid-derived metabolites from gut lactic acid bacteria induce differentiation of anti-inflammatory M2 macrophages through G protein-coupled receptor 40. *FASEB J.* **32**, 304–318 (2018).
291. Lee, S. J. *et al.* Mannose Receptor-Mediated Regulation of Serum Glycoprotein Homeostasis. *Science* **295**, 1898–1901 (2002).
292. Wynn, T. Cellular and molecular mechanisms of fibrosis. *J. Pathol.* **214**, 199–210 (2008).
293. Catarzi, S. *et al.* Oxidative state and IL-6 production in intestinal myofibroblasts of Crohn's disease patients: *Inflamm. Bowel Dis.* **17**, 1674–1684 (2011).
294. Yao, C. *et al.* Toll-like receptor family members in skin fibroblasts are functional and have a higher expression compared to skin keratinocytes. *Int. J. Mol. Med.* **35**, 1443–1450 (2015).

295. Zidar, N. *et al.* Pathology of Fibrosis in Crohn's Disease—Contribution to Understanding Its Pathogenesis. *Front. Med.* **7**, (2020).
296. Kanazawa, S. *et al.* VEGF, basic-FGF, and TGF-beta in Crohn's disease and ulcerative colitis: a novel mechanism of chronic intestinal inflammation. *Am. J. Gastroenterol.* **96**, 822–828 (2001).
297. Dropmann, A. *et al.* TGF-β2 silencing to target biliary-derived liver diseases. *Gut* **69**, 1677–1690 (2020).
298. Frangogiannis, N. G. Transforming growth factor–β in tissue fibrosis. *J. Exp. Med.* **217**, e20190103 (2020).
299. Li, Z. & Bratlie, K. M. Fibroblasts treated with macrophage conditioned medium results in phenotypic shifts and changes in collagen organization. *Mater. Sci. Eng. C* **122**, 111915 (2021).
300. Mohr, A. *et al.* The Influence of Apremilast-Induced Macrophage Polarization on Intestinal Wound Healing. *J. Clin. Med.* **12**, 3359 (2023).
301. Hult, E. M., Gurczynski, S. J. & Moore, B. B. M2 macrophages have unique transcriptomes but conditioned media does not promote profibrotic responses in lung fibroblasts or alveolar epithelial cells in vitro. *Am. J. Physiol.-Lung Cell. Mol. Physiol.* **321**, L518–L532 (2021).

Notes

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