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TESIS DOCTORAL:

Human Intestinal immune and microbial signatures in Inflammatory Bowel Disease: A multidisciplinary approach to disease mechanisms

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Abbreviations

6-MP: mercaptopurine

aCD: active Crohn's disease

AhR: aryl hydrocarbon receptor

AIEC: Adherent-invasive *Escherichia coli*

AIM-V: Albumin Insulin Transferrin – Version V

ALDH: aldehyde dehydrogenase

ANOSIM: Analysis of similarities

ANOVA: analysis of variance

APC: antigen presenting cell

ATP: adenosine triphosphate

aUC: active ulcerative colitis

AZA: azathioprine

BA: bile acid

Baft3: basic leucine zipper ATF-like transcription factor 3

BMDC: bone marrow dendritic cell

BMDM: bone marrow-derived macrophages

BSA: Bovine serum albumine

BSH: bile salt hydrolases

BTLA: B and T lymphocyte attenuator

CCR: C-C chemokine receptor

CD(X): cluster of differentiation number X

CD(X)L: CDX ligand

CD: Crohn's disease

cDC: conventional dendritic cell

cDC1: type 1 conventional dendritic cell

cDC2: type2 conventional dendritic cell

CEACAM: carcinoembryonic antigen-related cell adhesion molecule

CRC: colorectal cancer

CTLA4: Cytotoxic T-lymphocyte-associated protein 4

CXCR3: receptor for the CX3C chemokine

DC: dendritic cell

DMSO: Dimethyl Sulfoxide

DNA: deoxyribonucleic acid

DSS: dextran sulphate sodium

DTT: dithiothreitol

EDTA: Ethylenediaminetetraacetic acid

ETBF: Enterotoxigenic *Bacteroides fragilis*

FAE: follicle-associated epithelium

FBS: fetal bovine serum

FCS: Foetal calf serum

FlowSOM: Flow Self-Organizing Map

FMO: Fluorescence minus one

FMT: Faecal microbiota transplantation

FODMAP: Fermentable oligosaccharides, disaccharides, monosaccharides, and polyols

FOXP3: Forkhead box P3

FSC-A: Forward Scatter – Area

FSC-H: Forward Scatter – Height

GALT: gut-associated lymphoid tissue

GI: gastrointestinal

GPR: G protein-coupled receptors

HBSS: Hank's Balanced Salt Solution

HLA: Human leukocyte antigen

IBD: Inflammatory bowel disease

ID2: Inhibitor of DNA binding 2

IEC: intestinal epithelial cells

IFN: interferon

Ig: Immunoglobulin

IL: interleukin

ILC: innate lymphoid cell

ILF: isolated lymphoid follicle

IRF: Interferon regulatory factor

JAK: Janus kinase

LPMC: lamina propria mononuclear cells

LPS: lipopolysaccharide

MACS: Magnetic-Activated Cell Sorting

mAPC: myeloid antigen presenting cells

MHC: major histocompatibility complex

MLN: mesenteric lymph nodes

MMPs: matrix metalloproteinases

MO-DC: monocyte derived dendritic cell

MO-MΦ: monocyte-derived macrophages

MTX: methotrexate

MΦ: macrophage

NFIL3: Nuclear factor interleukin 3

NLR: NOD-like receptors

NOD: nucleotide-binding oligomerization domain

PBMC: peripheral mononuclear blood cells

PBS: Phosphate-buffered saline

pDC: plasmacytoid dendritic cell

PERMANOVA: Permutational multivariate analysis of variance

PHA: phytohemagglutinin

PP: Peyer's patch

PRR: pattern recognition receptor

qCD: quiescent Crohn's disease

qUC: quiescent ulcerative colitis

RA: retinoic acid

RALDH: retinaldehyde dehydrogenase

RBPJ: Recombination Signal Binding Protein for Immunoglobulin Kappa J Region

RELB: RelB Proto-Oncogene

RNA: ribonucleic acid

RORγt: RAR-related orphan receptor gamma t

ROS: Reactive Oxygen Species

RPMI: Roswell Park Memorial Institute medium

RT: room temperature

S1P: Sphingosine-1-phosphate

SA-PE: streptavidin-phycoerythrin

SCFA: short chain fatid acid

SES-CD: simplified endoscopic activity score for Crohn's disease

SIRPα: Signal regulatory protein alpha

SOCS: suppressor of cytokine signaling

STAT: signal transducers and activators of transcription

TCR: T cell receptor

TGFβ: Transforming growth factor-beta.

Th: T helper lymphocyte

TLR: Toll-like receptor

TNF: Tumor necrosis factor

Treg: regulatory T cell

UC: ulcerative colitis

UCEIS: Ulcerative Colitis Endoscopic Index of Severity

UMAP: Uniform Manifold Approximation and Projection

USA: United Stated of America

XCR1: X-C motif chemokine receptor 1

Generative artificial intelligence has been used for orthographical correction, translation and style enhancement in some paragraphs. All content has been critically reviewed and validated by the author to ensure accuracy and scientific relevance.

Chapter 1. Introduction

1.1 GENERAL OVERVIEW OF INFLAMMMATORY BOWEL DISEASE

Inflammatory bowel disease (IBD), which comprises ulcerative colitis (UC) and Crohn's disease (CD), is a chronic disorder of unknown aetiology characterized by an abnormal immune response, leading to chronic inflammation of the gastrointestinal (GI)-tract (1). IBD affects approximately 7 million people globally and its prevalence is continuously increasing and is projected to reach approximately 1% in some industrialized regions, including Europe and North America, within the next decade (2). At the turn of the 21th century, IBD has emerged as a global health concern, with its incidence rising rapidly in newly industrialized nations where societies have adopted westernized lifestyles (3), ranging from 10 to 30 per 100,000 in the Western world. In general, the prevalence of UC is higher than that of CD in Europe, whereas the reverse is observed in Australia. In North America, both conditions are distributed equally (Figure 1.1) (4).

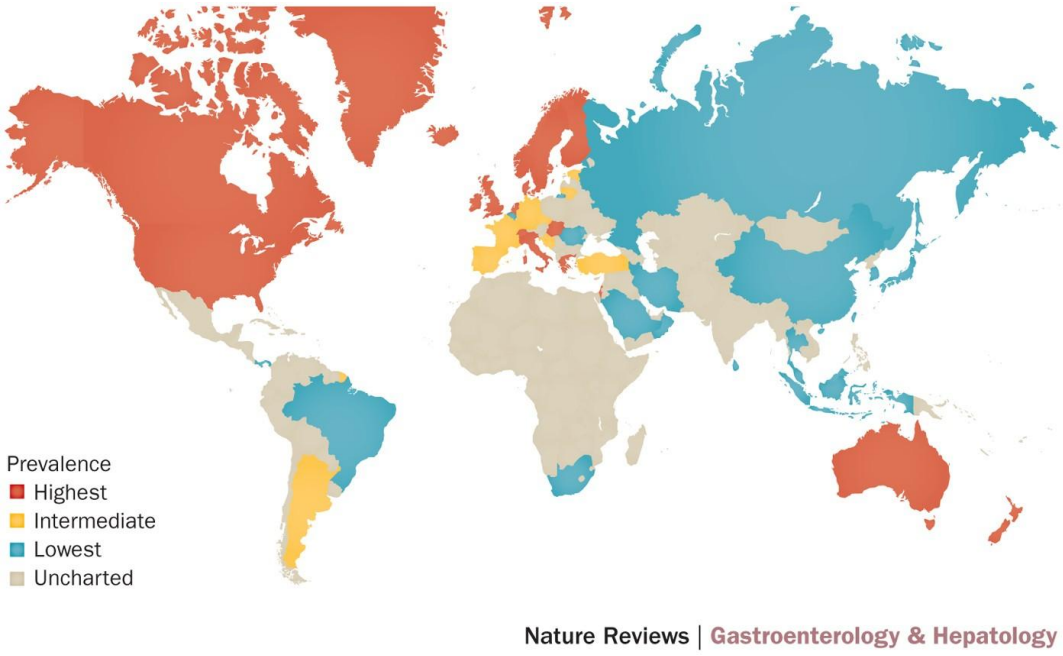

Figure 1.1.The global prevalence of IBD in 2015 *(4).*

The economic burden of IBD in the Western world is substantial, encompassing both direct and indirect costs. In the United States, it is estimated that over 2.4 million individuals are affected by IBD, with direct medical expenses surpassing $50 billion. Similarly, in Canada, more than 300,000 people are estimated to have IBD, resulting in annual direct healthcare costs exceeding CAD $3.3 billion (2). In Europe, about 3 million patients have IBD, and the direct health-care cost is estimated to be more than €5 billion annually (4).

Both CD and UC are chronic diseases characterized by relapsing and remitting periods and despite that their symptoms are similar, CD and UC have differences in their anatomical location, inflammation pattern and distribution (1). Whereas, CD can affect any area of the GI-tract, from mouth to anus, UC is confined to the large intestine (colon and rectum) (5,6). CD associated inflammation is heterogenous (known as “patchy inflammation”) and transmural so it could affect any layer of the intestinal wall whereas in UC the inflammation is continuous and is primarily confined to the mucosal and to a lesser degree, the submucosal compartments (1,5). The classic hallmarks of CD include abdominal pain, watery diarrhea, and weight loss, and the major complication associated to CD is the obstruction of the GI-tract (due to the formation of obstructive strictures) that could lead to an intestinal resection (5,7). On the other hand, UC symptoms comprise bloody diarrhea, abdominal pain and rectal bleeding (5) and major complications includes the development of toxic megacolon or even the development of colorectal cancer (CRC) (6,8,9).

IBD complexity has made challenging for traditional scientific methods to address key clinical questions. A complete understanding of its pathogenesis has yet to be achieved, indeed, current treatments remain far from optimal and currently IBD has no cure. Nevertheless, the therapeutic landscape for IBD is significantly evolving with the development of novel treatment options including targeted monoclonal antibodies and small molecules (8). Anti-tumor necrosis factor (anti-TNF) agents, such as infliximab and adalimumab, were the first biologics approved for IBD and have remained fundamental in its treatment ever since (9,10). Additionally, integrin antagonists like vedolizumab, which specifically targets the α4β7 integrin to block lymphocyte migration to the intestine, provide a more selective approach to treating IBD (11). The IL-12/IL-23 pathway has also emerged as a critical therapeutic target, as it is implicated in the regulation of Th1 cell responses (12), with antibodies directed against the IL-12p35 and IL-23p19 subunits showing encouraging results. Anti-IL12/23 drugs, such as ustekinumab, which targets the shared p40 subunit, have proven effective in both inducing and maintaining remission (13). More recently, selective IL-23 inhibitors, such as risankizumab and mirikizumab, which target the p19 subunit, have demonstrated promising outcomes in clinical trials (14,15). Sphingosine-1-phosphate (S1P) receptor modulators (ozanimod, etrasimod) have also emerged as a promising class of oral therapies for IBD, particularly UC. These agents retain lymphocytes within lymphoid tissues, thereby reducing their migration to the inflamed intestinal mucosa and mitigating inflammation (16,17). Finally, small molecule inhibitors of Janus kinases (JAKs), particularly JAK1 and JAK3, have been developed as oral treatments for IBD, introducing a novel method of modulating the inflammatory response. Tofacitinib,

upadacitinib (JAK1/3 inhibitors), and filgotinib (selective JAK1 inhibitor) represent a new class of oral small molecules that disrupt the intracellular signalling of several cytokines, especially interferon (IFN)α/pSTAT5 and interleukin (IL)-6/pSTAT1, (18–21).

The most accepted hypothesis regarding IBD pathogenesis establishes that it is derived from an abnormal response of the mucosal immune system towards the commensals (22,23). Indeed, IBD is a complex, multifactorial disease. Factors underlying IBD include several factors, including environmental (i.e. diet, use of antibiotics, geography, smoking abit…), genetical (around 240 loci have been associated with IBD), abnormal immune responses (i.e. overreactive Th1/Th17 axis) and gut microbiota (microbial dysbiosis) (23–25). In this thesis, I will focus on two fundamental pillars of IBD: the immune system and the gut microbiota, due to their crucial role in disease pathogenesis. The interplay between these components is central due to the loss of immune tolerance toward the gut microbiota, a key event in the development and progression of the disease (22,23). Understanding the mechanisms underlying this dysregulation is essential for a better comprehension of the disease, which could lead to the identification of potential therapeutic targets and biomarkers, improving current diagnosis and therapeutic strategies.

1.2 IMMUNE SYSTEM IN THE GASTROINTESTINAL TRACT

1.2.1 Structure and function of the intestine

The small and large intestines form a continuous tubular structure internally lined with a single layer of columnar epithelium, extending from the stomach outlet to the anus. The small intestine originates at the pylorus and terminates at the ileocecal valve, which serves as the entry point to the large intestine. It is composed of three primary segments: the duodenum, positioned closest to the stomach, followed by the jejunum, and finally, the ileum. The large intestine begins at the cecum and progresses through the ascending (proximal) colon, transverse colon, descending (distal) colon, and rectum, ultimately ending at the anus (26).

The local immune system must adapt to and function within this constantly changing environment. Most immunological processes occur within the mucosa, which consists of the epithelium, the underlying lamina propria, and the muscularis mucosa—a thin layer of muscle situated beneath the lamina propria. The lamina propria is composed of loosely arranged connective tissue that provides structural support for the villus while also housing the mucosal blood supply, lymphatic drainage, and nervous innervation. Additionally, it contains numerous cells from both the innate and adaptive immune systems. Below the muscularis mucosa is the submucosa, a connective tissue layer that plays a crucial role in housing a network of parasympathetic nerves. This layer is followed by a thicker muscle layer, and finally, the serosa forms a dense fibrous covering that separates the intestine from the surrounding peritoneal cavity (26).

The different regions of the intestine have distinct physiological functions. The small intestine is where most of the nutrients are absorbed. For this reason, it is covered by a layer of microvilli that increases the surface available for digestion and, in which are embedded enzymes (to digest dietary components) and nutrient transporters. The ileum, is the end part in which bile salts and vitamin B12 are absorbed, contributing less to nutrition. On the other hand, the large intestine has minimal or no inherent digestive function, primarily serving to reabsorb water and expel undigested food residues. Additionally, it acts as the primary reservoir for trillions of commensal bacteria that colonize the intestine and play a crucial role in maintaining gut homeostasis. Small and large intestines are covered in their inner layer by the mucus, which coats the mucosa and consists in a gel composed by mucin glycoproteins that acts a physical barrier (26).

The gut-associated lymphoid tissue (GALT) consists of subepithelial lymphoid aggregates located within the mucosa and submucosa, distinguished by a specialized follicle-associated epithelium (FAE). This epithelium contains microfold cells (M cells), which are specifically adapted to capture and transport particulate antigens from the intestinal lumen to antigen presenting cells. GALT includes Peyer's patches (PP), isolated lymphoid follicles (ILFs) dispersed throughout the intestine, the mesenteric lymph nodes (MLNs), the vermiform appendix, and various diffuse immune cells. Peyer's patches and ILFs are linked to the MLNs through lymphatic vessels, where they play a crucial role in recognizing antigens and activating immune cells in the intestinal mucosa (27).

1.2.2 Human intestinal dendritic cells and their subsets

Dendritic cells (DCs) are the most potent antigen presenting cells (APCs) and in the GI-tract they control the balance between immunity toward pathogens and tolerance toward commensals (28,29) . DC precursors migrate from the bone marrow to most tissues in the body, including the mucosa in the GI-tract, where they become sentinels and sensors of the immune system (30). DCs can be divided into two major subsets: conventional or classic DCs (cDC) or plasmacytoid DCs (pDC) (23,31). pDCs are specialized in the production of type I interferons during viral infections and are critical in antiviral immune responses (32). cDCs are the professional APC of the innate immune system, hence will be the main focus of this thesis (28).

cDCs have a unique capacity to migrate to the lymph nodes and stimulate naïve T cells, that is, they can influence or "program" T cells to express specific molecules that guide them to particular tissues, ensuring that immune responses occur in the appropriate locations (22,33,34), as it is explained in detail in 1.2.4 section (oral tolerance). cDCs act also as sensors given their capacity to identify the nature of the antigen, discriminating between potentially harmless and harmful antigens via their high expression of pattern recognition receptor (PRR) molecules, including Toll-like receptor (TLRs) (30).

Intestinal cDC in the steady state are generally tolerogenic, as they produce IL-10 and promote the generation and maintenance of regulatory T cells (Tregs) (35,36). As their primarily function is to present the antigens to lymphocytes, cDCs are highly effective in stimulating both B and T lymphocytes in the MLNs. B cells can directly recognize native antigens through their B cell receptors. In contrast, T lymphocytes require the antigen to be processed previously. T cell receptors (TCRs) recognize antigen fragments bound to major histocompatibility complex (MHC) molecules on the surface of an APC. There are two types of peptide-binding proteins: MHC class I, which activate cytotoxic T cells, and MHC class

II, which activate helper T cells (37). The migration to the MLN occurs in a CCR7-dependent manner (38) and provokes DC maturation which is characterized by three primary changes or signals: The first signal involves an enhanced surface expression of processed antigens, facilitated by an increase in HLA molecules. The second signal is the upregulation of co-stimulatory molecules such as CD80/CD86, which are ligands for T cell CD28/CTLA4, as well as CD40, the ligand for T cell CD40L. The third signal is associated with a shift in cytokine production, which alters the balance between pro-inflammatory and regulatory cytokines (39). Once in secondary lymphoid tissues DCs are extremely efficient in antigen presentation and in stimulating T cells, true effectors of immune response (29).

In the intestine, cDCs are further divided into subsets based on their ontogeny and function: type 1 cDCs (cDC1) express CD103, and type 2 cDCs (cDC2) express CD172 (SIRPα) (40,41). Notably, the intestine harbours a unique subset of cDC2 which express both CD103 and SIRPα, which controls most of the mechanisms of immune tolerance given its unique capacity to generate gut-homing $CD4^+$ $FOXP3^+$ Tregs (by inducing the expression α4β7 and/or CCR9) and IgA-producing B cells, although they can also drive Th17 responses (42–44). cDC1 express the surface markers XCR1 and CD141 in human and while cDC2 express CD1c. In addition, human cDC1s requires IRF8, ID2, NFIL3 and Baft3 transcription factors for their development while RELB, RBPJ and IRF4 are transcription factors that identifies cDC2 (40,41). cDC1 are specialized in cross-presentation to cytotoxic $CD8^+$ T cells due to the expression of XCR1 (45). Among cDC subtypes, intestinal $CD103^+$ cDCs are thought to be the regulators of oral tolerance as they have the distinctive function of metabolizing vitamin A into retinoic acid (RA) through the activation of the retinaldehyde dehydrogenase (RALDH2) enzyme (28,46).The RA produced by these intestinal $CD103^+$ DCs plays a crucial role in regulating immune responses by imprinting gut-homing specificity on T cells, B cells, and innate lymphoid cells (ILCs). Additionally, RA has additional effects: induces the differentiation of IgA-producing B cells, promotes TGFβ-dependent differentiation of induced Tregs or suppresses the differentiation of Th17 cells (47) (see section 1.2.4). On the contrast, $CD103^-$ cDC2 may play a key role in driving effector T cell responses occur through the production of proinflammatory cytokines such as IL-6, TNF-α, IL-12, and IL-23 (28,48). Table 1.1 summarizes principal phenotypic and functional differences among human cDC subsets.

Table 1.1. Principal differences between human intestinal conventional dendritic cell subsets.

cDC subset	Surface markers	Transcription factors	Principal functions
cDC1	CD103, XCR1, CD141	IRF8, ID2, NFIL3, Baft3	Promote cytotoxic $CD8^+$ T cell response
cDC2 $CD103^+$	SIRPα,	RELB, RBPJ and IRF4	Regulate immune responses
cDC2 $CD103^-$	SIRPα,	RELB, RBPJ and IRF4	Drive effector T cells responses (Th1)

Plasmacytoid dendritic cells (pDCs) are present in the intestinal mucosa but in lower numbers than cDCs (26). Human pDC express the surface marker CD123 and the transcription factor E2-2 (41). Unlike cDCs, pDCs do not migrate to MLNs but may facilitate cDC mobilization into lymph in response to TLR7/8 ligands, through TNF and type I IFN production (38,49). pDCs also contribute to immune tolerance and play protective roles in models of small intestinal inflammation and food allergy (50). For example, polysaccharide A from *Bacteroides fragilis* may modulate immune responses by activating TLR2 on pDCs, promoting IL-10-producing CD4+ T cells (51).

Most studies involving intestinal DC are based on mouse models, indeed research regarding human intestinal DC is limited, primarily due to methodological difficulties in the isolation of human gut DC. It is known that mice DC have markers that are shared with humans, but others are different. Classically, DC has been defined in mice as $CD11c^{high}MHC\text{-}II^+$ cells. pDC and cDC can be differentiated also in mice: B220 and Siglec H allows to identify mice pDC whereas surface markers BTLA, CD117 characterizes cDC. Within cDC, mice cDC1 express CD103 and XCR1 as occurs in human DCs, but also CD8α. Mice cDC2, on the contrary, express CD172 /SIRPα like human cDC2 and CD11b and CX3CR1 (40). It is also interesting that as occurs in human, $CD103^+$ cCDs have been found in murine MLNs to confer gut-homing markers CCR9 and β7 on responding T cells, suggesting a conserved mechanism between the species (52,53). However, in mice another population of cDC ($CD103^-CX3CR1^{int}$ DCs) also migrate to the MLNs and prime effector T cells. These cells induced differentiation of IFNγ and IL-17-producing effector T cells suggesting a role in generation of inflammatory T cell responses (54).

1.2.3 Human intestinal macrophages

Macrophages (MΦ) are the most abundant antigen presenting cells in the healthy intestinal lamina propria where they serve as the first line of defense against invading pathogens

(26). MΦs are strategically located in the subepithelial area where they regulate lumen-derived commensal microbe penetrance through their capacities of phagocytosis and degradation (55). As such, they exhibit high phagocytic activity and potent bactericidal properties (56,57). Interestingly, despite their continuous exposure to microbiota and their byproducts, these cells do not trigger an inflammatory response due to their unresponsive phenotype, a phenomenon known as inflammatory anergy (57). This phenotype is primarily induced by the tolerogenic environment of the lamina propria, which is mediated by elevated levels of interleukin-10 (IL-10) secreted by tissue-resident $CD4^{+}FOXP3^{+}$ Tregs (58–60).

The crucial importance of MΦ as major gatekeepers is that they rapidly adapt their function by sensing the surrounding microenvironment and acquiring a specific phenotype based on the microanatomical niche they occupy (61). This occurs in the GI-tract, where they play a crucial role in maintaining intestinal homeostasis by phagocytosing and degrading microorganisms and dead cells, as well as producing mediators that promote epithelial cell renewal. Additionally, they secrete large amounts of IL-10, which inhibits pro-inflammatory responses to stimuli such as TLR ligation and supports the survival and function of $FOXP3^{+}$ Tregs in the mucosa (59,60) and their response to IL-10 is also essential for preserving local immune balance. Moreover, the production of IL-1β by resident macrophages in response to the microbiota may contribute to sustaining Th17 cell activity in the steady-state small intestine (62).

Characterization of human MΦ can be performed using CD14, CD64 and CD163 (55). However, regarding their origin, most of the current understanding of MΦ biology has been derived from murine models, which have shown that GI-Mϕ, unlike those from other tissues that typically originate from yolk sac or fetal liver precursors, are continually replenished by circulating $Ly6C^{high}$ monocytes that enter the GI mucosa in a CCR2-dependent manner (56,63). Once these $Ly6C^{high}$ monocytes arrive, they are conditioned by the tissue microenvironment through several intermediates via the "monocyte waterfall" (56,63), leading to the differentiation of tissue-resident tolerogenic macrophages ($Ly6C^{-}MHCII^{high}CX3CR1^{high}CCR2^{-}$) (56,64). In humans, a similar “monocyte waterfall” occurs. Human intestinal Mϕ can be divided into subsets based on the expression levels of the CD11c integrin and the chemokine receptors CCR2 and CX3CR1. Hence, proinflammatory monocyte-like cells can be identified as $CD11c^{high}CCR2^{+}CX3CR1^{+}$ cells, a phenotype also shared by circulating $CD14^{+}$ monocytes. On the contrary, tissue-resident tolerogenic Mϕ can be identified as $CD11c^{-}CCR2^{-}CX3CR1^{-}$ cells. Moreover, a transition phenotype between the two can be also found based on CD11c expression with such a subset displaying an intermediate phenotype and function between the others (64).This

phenotype comprises newly arrived monocytes or immature Mϕs, whose maturation process comprised a decrease in the expression of some blood monocyte markers such as CD11c and CCR2, as well as an increased expression of CD163 and CD209 (65). Mature Mϕs reduce the release of proinflammatory molecules together with desensitization to TLR ligands, which is a functional feature of anergy (65). This anergic status is thought to be driven by several micro-environmental factors, such as TGFβ, which induces downregulation of the MyD88 pathway in blood monocytes and results in tolerogenic Mϕs (66).

It is now well stablished that human intestinal MΦs comprise a continuum of blood monocyte-derived cells differentiating into immature MΦs. Differentially, monocytes originate in the bone marrow from common monocyte progenitors, which derive from common myeloid progenitors. In healthy individuals, they represent 2–8% of leukocytes in peripheral blood and constitute a dynamic and versatile cell population, comprising three main subsets: classical ($CD14^+CD16^-$), intermediate ($CD14^+CD16^+$), and non-classical monocytes ($CD14^-CD16^+$). Classical monocytes circulate in the bloodstream for only one day before migrating to peripheral organs, where most of them differentiate into tissue-resident Mϕs and, potentially, DCs -although this is a matter of discussion- in response to growth factors, cytokines, and microbial products in the local microenvironment. Each of these subsets has specific extravasation and cell properties, been implicated in different diseases (55).

1.2.4 Oral tolerance

The intestine contains the largest number of immune cells of any tissue in the body as it is continually exposed to a wide range of antigens and potential immune stimuli (26). One of the most well-known and distinctive characteristics of the intestinal immune system is its capacity to establish tolerance to the vast array of harmless foreign antigens it regularly encounters while simultaneously developing an active immune response against pathogens (34,67,68).

Oral tolerance is a crucial immunological process that prevents excessive immune responses to harmless antigens encountered in the GI-tract. This process occurs in several stages. Summarizing, first, antigens must be transported from the intestinal lumen to the underlying immune cells located in the lamina propria or GALTs. Once there, APCs capture these antigens, enabling their processing, transportation, and presentation to specific T cells. Finally, the interaction between APCs and T cells, influenced by signals from both the

APCs and the local microenvironment, drives the differentiation of T cells into Tregs, characterized by a tolerogenic phenotype (67,68).

The induction of oral tolerance primarily relies on the function of cDCs, which capture and present luminal antigens to promote the differentiation of Tregs from naïve $CD4^+$ T cells. This process occurs mainly in the MLNs, where migratory cDCs transport antigens from the intestinal lamina propria through a CCR7-dependent mechanism (69–71). Among cDC subsets, cDC1 and cDC2 play complementary roles in oral tolerance: while cDC1 cells can induce a population of tolerogenic $CD8^+$ T cells through the combined action of TGFβ, RA, and PD-L1 (43), cDC2 cells are more efficient at presenting soluble antigens via MHCII to $CD4^+$ T cells (49,72). The differentiation of Tregs in this context is facilitated by key factors produced by cDCs, including TGFβ and RA (73–75). Intestinal cDCs express αvβ8 integrin, which activates latent TGFβ, and aldehyde dehydrogenases (ALDH) and retinaldehyde dehydrogenases (RALDH), which convert dietary vitamin A-derived retinol into active RA, thereby reinforcing their tolerogenic properties (69,70). Local factors, such as retinoids derived from the diet or secreted in bile, promote the expression of these RA-generating enzymes in intestinal cDCs, while TGFβ signaling drives the expression of αvβ8 integrin, essential for TGFβ activation (76). The gut microbiota further influences this process by stimulating intestinal epithelial cells (IECs) to release retinoids that enhance RA production by cDCs, increasing their capacity to induce Tregs in the MLNs. Following the migration of intestinal cDCs to the MLNs, stromal-derived TGFβ and RA further enhance their Treg-inducing potential (77) (Figure 1.2) (68).

In addition to cDCs, lamina propria Mϕs also play an indirect role in oral tolerance and Treg cell generation through IL-10 production. In humans, the high expression of β8 integrin by intestinal Mϕs suggests that they may also contribute to local TGFβ activation, thereby reinforcing the differentiation and stability of Tregs within the intestinal mucosa (68,78).

Tregs involved in oral tolerance primarily arise from naïve $CD4^+$ T cells that, upon encountering luminal antigens in the gut, differentiate into peripherally induced Tregs (pTregs) expressing the transcription factor FOXP3 (79). It was suggested that most Tregs involved in oral tolerance are generated in the periphery rather than in the thymus. Despite their rapid turnover within the intestinal mucosa, oral tolerance remains long-lasting, even after a single antigen exposure. This persistent tolerance may be attributed to the presence of long-lived memory Tregs in secondary lymphoid organs, which continuously replenish effector Tregs in the intestinal mucosa and prevent the activation of immune responses against dietary antigens (34,80,81) (Figure 1.2) (68).

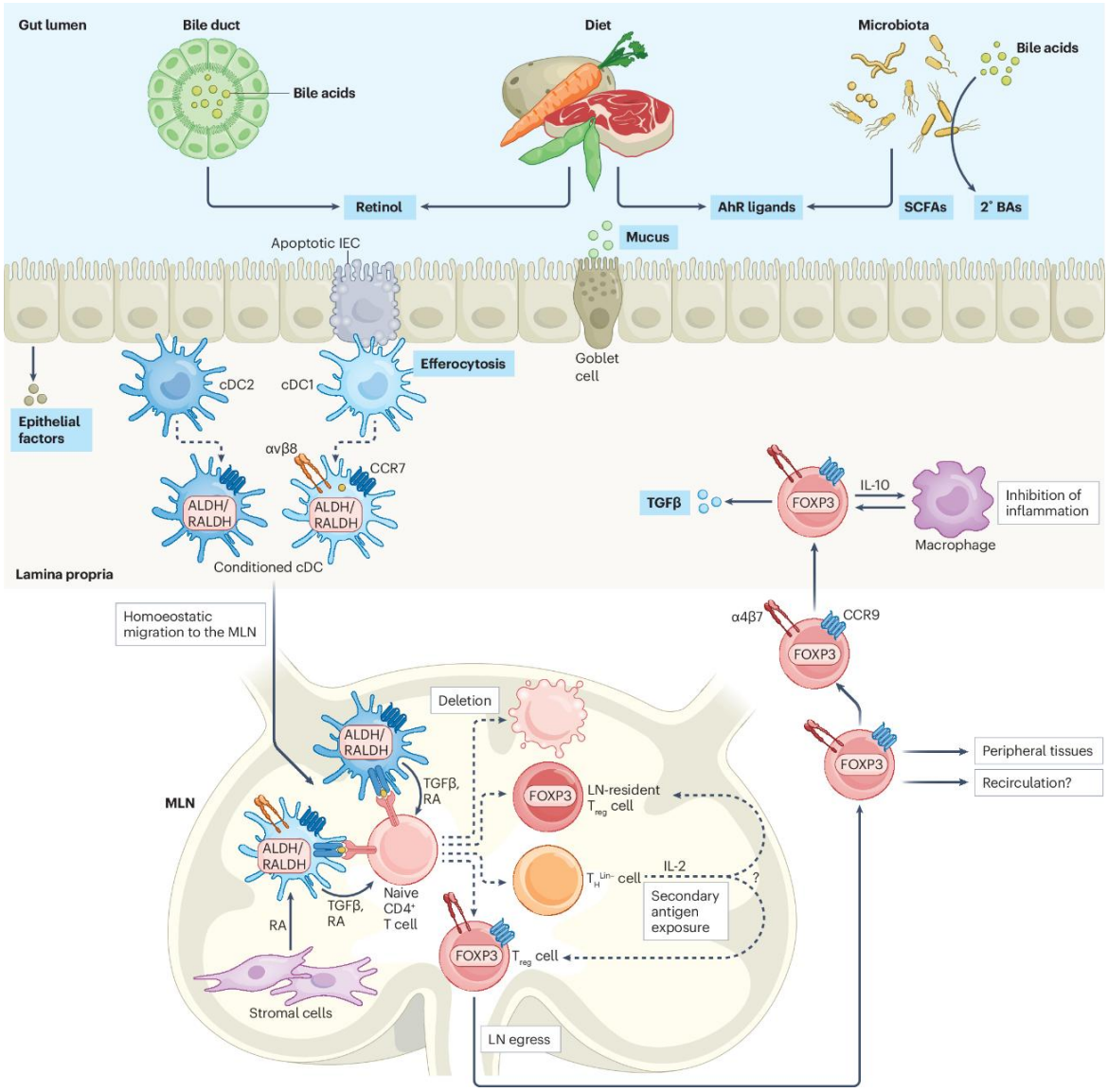

Figure 1.2. Mechanisms underlying oral tolerance *(68).*
Under steady-state conditions, intestinal dendritic cells (DCs) acquire a tolerogenic phenotype through conditioning by dietary retinoids, microbial metabolites, and host-derived factors such as TGFβ and IL-10. These conditioned DCs migrate to mesenteric lymph nodes (MLNs), where they further mature and induce FOXP3⁺ regulatory T cells (Tregs) via retinoic acid and TGFβ activation. Tregs then home to the intestine, where they are maintained by macrophage-secreted IL-10, or remain in lymph nodes to limit further immune activation. Additionally, antigen-specific T cells may undergo deletion or adopt an anergic phenotype, reinforcing immune tolerance.

1.3 DYSREGULATION OF THE IMMUNE RESPONSE IN IBD

The most accepted hypothesis regarding IBD pathogenesis establishes that is derived from an abnormal response of the mucosal immune system towards the commensals, driven by APCs including DCs and Mϕs, and subsequently mediated by pro-inflammatory T cells (22,23,82). The loss of oral tolerance leads to a waterfall of mechanisms that exacerbate the activation of the immune system.

The innate immune response is the body's first defence against pathogens. The barrier function of IECs is established by a network of tight junctions between them, preventing the passage of substances from the lumen. Besides this epithelial barrier, some IEC, like Goblet cells, produce mucins that form the mucus layer on the apical membrane of the IEC. This mucus layer is rich in antimicrobial defensins, neutrophils, and secreted IgA, contributing to the protection of the GI-tract (83). Innate immune cells, including DC and MΦ, are found in the lamina propria underneath the IEC. Both DC and MΦ recognize pathogen associated molecular patterns (PAMPs) by their PRRs. PRRs include transmembrane TLRs and intracellular receptors such as nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). Activation of PRR signalling pathways leads to nuclear factor (NF)-κB activation, initiating gene transcription and the production of pro-inflammatory mediators that support an effective innate immune response. Additionally, PRR stimulation drives the maturation of APCs, enhancing the expression of co-stimulatory molecules essential for efficient antigen presentation and T cell activation (82). Indeed, NOD2, from the NLR family, was the first identified gene that confer increased risk to CD (84,85).

The key role of APCs in linking innate and adaptative immune responses as well as determine the kind of response (tolerogenic or inflammatory) makes them pivotal players in IBD pathogenesis, for this reason the next section will focus on APC dysregulation in IBD.

1.3.1 Role of cDC, macrophages and monocytes in inflammation

In healthy conditions, the immune system in the GI-tract promotes immune tolerance against commensals and food, by the generation of antigen specific Ig-A secreting B cells and T cells with regulatory properties. However, in IBD, the immune system fails and drives the development of pro-inflammatory T cells, which control the progression of the disease. In the human health intestine, conventional dendritic cells (cDCs) exhibit a tolerogenic profile whereas in IBD, they display a pro-inflammatory profile (28,30,35,86). The inflamed mucosa from IBD patients have reported increased numbers of DC, and more specifically,

a decrease in the $CD103^+$ subset (87,88). Indeed, DC from IBD patients increased its expression of several pattern recognition receptors (like TLRs), increasing their capacity to recognize microbial antigens and, therefore, exacerbating immune responses (35). Consequently, cDCs from IBD patients have a reduced capacity to generate immunosuppressive regulatory T lymphocytes while together with an increased ability to differentiate pro-inflammatory Th1 and Th17 effector lymphocytes (86). This phenomenon is presumed to result from a deficiency of $CD103^+$ cDCs (86,89,90).

The phenotype and function of DCs are primarily shaped by their surrounding microenvironment (91). Consequently, in a colonic environment, DC develop a tolerogenic "gut-like" profile which is abrogated in IBD due to the pro-inflammatory cytokine environment (92). Thus, the altered phenotype and function observed in intestinal DC from IBD patients are likely a result of the persistent inflammation (30). Under normal conditions, IEC release regulatory signals, such as IL-10 or RA, which promote a tolerogenic phenotype in DC (93). However, when danger signals are present—such as during an infection—IEC cease producing these "sedative" signals, preventing them from maintaining DC in a regulatory state. This shift occurs because IEC can recognize bacteria at their apical membrane via PRRs and respond by secreting TGF-β and RA, however, when pathogenic bacteria disrupt tight junctions and penetrate the epithelial layer, they engage PRRs on the basolateral membrane instead (94,95). In this scenario, IEC stop producing inhibitory signals and, as a result, DC interpret the antigens they capture as pathogenic invaders rather than harmless components of the gut, leading to the suppression of immune tolerance and the activation of an immune response (30). This adaptability allows DC to swiftly adjust to their surroundings, playing a crucial role in balancing immune activation and tolerance.

As it is mentioned above, GI-Mϕs serve as a primary phagocytic defense against invading antigens and regulate effector T cell responses within tissues (96). They remain unresponsive to commensal microbiota, thereby preserving tissue homeostasis (22,68,97). However, despite this tolerogenic function, the frequency of Mϕ among intestinal lamina propria is increased in IBD patients compared to controls, especially in active lesion areas. Those Mϕ also present an immature phenotype, and consequently, less tolerogenic (98–100). This accumulation in IBD patients seems to result from the inflammatory intestinal microenvironment, which enhances the recruitment of classical monocytes through mechanisms involving CCL2, IL-8, and TGF-β signalling (98,101). These newly recruited monocytes remain in an immature pro-inflammatory state, further exacerbating chronic intestinal inflammation (102,103). In addition, intestinal Mϕs produced more pro-inflammatory cytokines, such as TNF, IL-23, IL-1β and IL-6, in basal conditions as well as

after TLR stimulation, in UC patients and even more in CD patients compared to controls, promoting a pathologic environment (89,100,102). Notably, Mϕs from CD patients also showed to express higher levels of both IL-10 and latent TGF-β, which have anti-inflammatory effects (89,104). However, the expression of integrin αvβ8, which regulates immune tolerance through TGF-β activation, is significantly reduced in Mϕs from IBD patients, suggesting that the amount of active TGF-β is lower (78). Other disease-associated changes in Mϕ function that may promote IBD is their contribution to intestinal barrier dysfunction. In this way, Mϕs from inflamed CD tissue trigger less IL-22 secretion by ILCs than those from non-inflamed areas (105) and produce more ROS, contributing to epithelial damage (106). Moreover, blood monocytes alter the structure and integrity of tight junctions, compromising epithelial barrier function (107). However, it remains uncertain whether the impaired differentiation of blood monocytes into mature Mϕs is due to the absence of intrinsic maturation signals that typically guide recruited monocytes toward tolerogenic Mϕs or if to the introduction of new factors derived from the chronic inflammatory microenvironment that actively disrupt this homeostatic process.

1.3.2 Role of Th1, Th2, and Th17 cells

As opposed to the innate immune response, the adaptive immune system is highly specific having T cells a central role on its function. An imbalance in T cell responses, characterized by abnormal activation and differentiation of T cell subsets, can trigger inflammation through excessive cytokine and chemokine release. These molecules exert different effects on both the adaptive and innate immune systems (82). Th1 cells, driven by IL-12, produce IFN-γ, while Th2 cells release IL-4, IL-5, and IL-13 (106). In CD, an exaggerated Th1 response, triggered by elevated IL-12 and IL-18 levels, is believed to contribute to intestinal inflammation (107). CD patients show also increased IL-2 and IFN-γ production in mucosal T cells compared to UC patients and healthy controls (110). For this reason, CD has been traditionally considered a Th1-driven disease, while UC has been linked to a Th2 response with excessive IL-5 and IL-13 production. However, this classification remains controversial as IL-13 has been found decreased in both CD and UC biopsies in several studies even suggesting an anti-inflammatory role for IL-13 (111–113).This also led to a controversial role for Th2 cells in IBD. IFN-γ produced by Th1 cells induces enterocyte apoptosis and stimulates activated mucosal Mϕs to release TNF-α. Th1 cells are also a major source of TNF-α, which is central to the differentiation of stromal cells into myofibroblasts which produce matrix metalloproteinases (MMPs), a group of enzymes that degrade tissue and contribute to enterocyte apoptosis (114). TNF-α thus serves as a key mediator connecting

the innate and adaptive immune responses, playing a critical role in the pathogenesis of IBD and is an important target in biological IBD treatments.

Th17 cells are a T cell subset characterized by the production of large amounts of IL-17A, IL-17F, IL-21 and IL-22 (115). In addition, Th1/Th17 cells release both IFN-γ and IL-17A (116,117). Th17 cells are induced by a combination of IL-6 and TGF-β, and their expansion is promoted by IL-23 (115). IL-21 produced by Th17 cells in turn increases their expression of the IL-23 receptor, therefore potentiating the expansion of this cell subtype by a positive autoregulatory feedback loop (118). Supporting this, high transcript levels of IL-17A have been detected both in CD and UC mucosa in comparison to normal gut, and it has been observed that IL-17A is overexpressed in the lamina propria of IBD patients (119–121). Th17 cells also serve as a key source of IL-21, a cytokine related to IL-2, which is overexpressed in inflamed IBD mucosa and promotes Th1 and Th17 immune responses in the gut (122,123). Several functions have been demonstrated for IL-17A; for instance, IL-17A recruit neutrophils to the site of inflammation and upregulates several pro-inflammatory molecules, such as inducible nitric oxide synthase and IL-1β (124). IL-17 can also stimulate the proliferation of IECs, enhance intestinal IgA secretion, and promote the release of antimicrobial peptides, all of which contribute to the healing of intestinal mucosal injury and improve the intestinal barrier function. By binding to receptors on Th1 cells, IL-17 also suppresses the secretion of IL-23R, IFN-γ, IL-12Rp2, and other pro-inflammatory factors, thereby inhibiting their immune regulation. This dual nature of IL-17A makes it an ineffective target for IBD.

1.4 ROLE OF JAK-STAT SIGNALING PATHWAY IN IBD

The Janus kinase (JAK)–signal transducer and activator of transcription (JAK-STAT) pathway is a highly conserved signaling mechanism across evolution, playing a fundamental role in various essential physiological processes such as hematopoiesis, cellular differentiation, metabolism, and immune regulation (125,126). Indeed, more than 50 types of cytokines, including interferons (IFNs), interleukins (ILs), and growth factors, have been shown to play roles in JAK-STAT signaling contributing to different physiological processes (127–129).

1.4.1 The JAK family

The structure of the JAK-STAT pathway includes transmembrane receptors, receptor-associated cytosolic tyrosine kinases (JAK), signal transducers and activators of transcription (STAT) (130). The JAK protein family contains four members: JAK1, JAK2, JAK3, and TYK2 (131,132). Each kinase functions as an intracellular adaptor protein for cytokine signaling (133,134). Cytokines such as interferons, interleukins, growth factors and their receptors are the main activators of JAK (135). The receptor-ligand complex triggers the activation of JAK proteins associated with the receptor, leading to the phosphorylation of a receptor tyrosine. Every JAK family member interacts with distinct cytokine receptors, facilitating the recruitment of specific STAT proteins to mediate several biological functions (136–139). JAK1, JAK3, and TYK2 play key roles in the development and regulation of the immune system, while JAK2 is primarily involved in hematopoiesis (138,139).

1.4.2 The STAT family

STAT proteins are signaling molecules downstream of JAK. The STAT family consists of seven proteins: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6 (140,141). The mechanism of action starts when the receptor binds to an extracellular ligand (i.e. a cytokine) and then JAKs initiate the phosphorylation of the tyrosine of the receptors and recruit corresponding STAT (127,128). This phosphorylated STAT then dimerizes and reach the nucleus to regulate specific gene transcription. This process ensures the rapid transmission of external signals to the nucleus to regulate biological and pathological processes (142). Once the receptor tyrosine is phosphorylated cytosolic STAT are recruited to the activated receptor, and a STAT tyrosine is phosphorylated, leading to the formation of STAT dimers (143,144). STAT dimers then enter the nucleus as a component of

transcription factor complexes to promote the transcription of specific genes (145). STAT proteins are then dephosphorylated and return to the cytoplasm. Its role as a key mediator in transmitting signals from the plasma membrane to the nucleus makes it a promising target for drug development (146,147).

1.4.3 The JAK-STAT pathway in immunoregulation

Cytokines are key players in humoral and cellular responses, (148,149). Interactions among numerous cytokines and the JAK-STAT pathway play a pivotal role in immune cell differentiation and development, contributing to immunoregulation. IFN-γ and IL-12 are essential for Th1 cell differentiation, promoting T-bet gene expression through STAT1 and STAT4, respectively. IL-4 enhances GATA3 gene expression via STAT6, facilitating Th2 cell differentiation. IL-6 and TGF-β are critical for Th17 cell differentiation, activating RORγt expression through STAT3. Additionally, IL-6 and IL-12 regulate T follicular helper cell differentiation via STAT3 by increasing Bcl-6 transcription. IL-2 drives Treg cell differentiation by directly interacting with STAT5A/B to activate the Foxp3 gene (150,151). Indeed, the JAK-STAT pathway has been implicated in several autoimmune diseases such as IBD, rheumatoid arthritis or lupus erythematosus (152). JAK-STAT implication in IBD will be deeper described in the next section (1.4.4).

1.4.4 JAK-STAT pathway in IBD

Cytokines such as IL-6, IL-10, IL-2, and IL-22, as well as those known to drive pathological responses in UC and CD, including IFN-γ, IL-12, IL-23, and IL-9, rely on JAK-STAT-mediated signalling. JAK and STAT proteins are utilized by a wide range of cytokine receptors, and their expression is not necessarily confined to specific cell types. However, JAK3 is an exception due to its exclusive association with the common cytokine receptor Ɣ chain (Ɣc) containing cytokine receptors, which include IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 (153). Signalling through the Ɣc receptor is essential for lymphocyte development, maintenance, and function (154). Indeed, JAK-3 deficient individuals exhibit abnormalities in lymphoid compartments but not in myeloid function (155).

Although it is evident that active IBD is linked to an increased transcription of JAKs, the specific cell types that predominantly express each JAK protein and those responsible for regulating their expression in inflamed human tissues remain poorly characterized. Although there is little published data, evidence collectively indicates that JAK expression is compartmentalized at the cellular level and suggests that different cell subsets and/or

cytokine pathways may contribute distinctly to intestinal homeostasis. The expression of JAKs in the inflamed mucosa is also likely to be differentially regulated within these distinct cell types (157). For example, high characteristic expression of JAK3, among other proteins, was observed in a recently identified subset of inflammatory fibroblasts that are enriched in active UC but not in the healthy mucosa (159). During inflammation, a significant alteration of cells and functions highly dependent on JAK signalling is likely to play a crucial role in the inflammatory process.

In the same way, the implication of the STAT family on IBD is also being investigated. Important implications of STAT proteins have been shown, for instance, STAT1 is phosphorylated by either JAK1 and JAK2 or JAK1 and TYK2 and has a fundamental role in signalling via the IFN-γ and related family of receptors (157). Research findings on STAT1 expression in IBD have been somewhat contradictory. Some studies report increased expression and activation in both UC and CD, while others indicate that total STAT1 levels are higher in CD compared to healthy controls, but without a corresponding increase in its phosphorylated (active) form—a pattern not observed in UC (160,161). Although the exact role of STAT1 in intestinal inflammation remains uncertain, its activation appears to have different effects depending on the cell type. In lymphocytes, STAT1 activation promotes pro-inflammatory responses, while in MΦs and IECs, it seems to contribute to protective functions (163,164). STAT2 is involved in signal transduction in response to type I interferons and has been studied in the context of IBD, since one study has suggested that STAT2 is downregulated in IBD (161). Similarly, STAT3 is the most widely studied STAT protein and seems to have a fundamental role in IBD, but its protective or anti-inflammatory potential remains elusive. STAT3 activation is also essential for cellular responses to IL-10 family members such as IL-10 and IL-22 (potentially anti-inflammatory cytokines), however several studies have reported an increased expression of STAT3 or STAT3 phosphorylation in human IBD (161,162,165). In addition, STAT3 has been also shown to be essential for the differentiation of Th17 cells and for Th17 cell-dependent murine colitis (164–166). Data regarding STAT4 is better known. STAT4 phosphorylation is driven by JAK2 and TYK2 in response to cytokines such as IL-12 and IL-23. STAT4 signalling plays a critical role in Th1 cell development in response to IL-12 (167,168), in regulating IFN-γ expression in natural killer cells (169), and in Th1 cell activity induced by IL-21 (123). Furthermore, STAT4 has been implicated in IL-23-driven responses in memory Th17 cells (170). Given the well-established involvement of the IL-12, IFN-γ and IL-23 pathways in IBD, targeting STAT4-mediated signalling represents a potential therapeutic strategy (156). On the other hand, STAT5 activation primarily occurs through JAK1 and JAK3 following stimulation of Ɣc family receptors. Additionally, STAT5 can be activated in

response to IL-3 and the single-chain cytokine family, including growth hormones (156). In line with these findings, STAT5 has been shown to stimulate the proliferation of IECs, a process crucial for intestinal crypt regeneration (171). Furthermore, STAT5 plays a key role in IL-2-dependent FOXP3 induction, which is essential for Treg cell differentiation, and in redirecting Th17 cell differentiation towards a Treg phenotype (172). All these studies support the idea that STAT5 is a key regulator of intestinal epithelial regeneration and immune balance, promoting epithelial proliferation and Treg cell differentiation while modulating Th17 responses. Similarly, STAT6 phosphorylation is also mediated by JAK1 and JAK3; however, the activation is triggered specifically through the Yc receptors IL-4R and IL-13R (173). Although its primary role is to induce Th2 type responses, to our knowledge there is currently no data supporting a clinical benefit of inhibition of the Th2 cell response mediated by IL-13 (174,175).

1.4.5 JAK-STAT inhibitors for IBD treatment

Due to its essential role in cytokine signaling directly associated with various cancers and autoimmune diseases, the JAK-STAT pathway has become a significant target for drug development. Drugs targeting this pathway can be classified into three main categories based on their impact on signal transduction: cytokine or receptor antibodies, STAT inhibitors, and JAK inhibitors (129,176,177).

Therapeutic agents that modulate JAK-STAT-dependent cytokines and receptors, such as siltuximab and tocilizumab, which block IL-6 signaling, can inhibit JAK-STAT signal transduction and have been utilized in the treatment of various diseases (178).

On the other hand, most STAT inhibitors act by preventing STAT phosphorylation, blocking its dimerization, or promoting STAT degradation (179,180). Given the vital role of activated STAT3 and STAT5 in signal transduction and disease progression, several inhibitors specifically targeting STAT3 and STAT5—such as peptides, peptidomimetics, oligonucleotides, siRNAs, small molecules, and metal-based complexes—have demonstrated promising efficacy in preclinical studies (181,182).

Additionally, suppressor of cytokine signalling (SOCS) proteins, which function as part of a negative feedback loop in the JAK-STAT pathway (by blocking JAK proteins), and peptides targeting SOCS interactors have also shown potential as inhibitors of disease progression (183,184). JAK inhibitors, which are small-molecule compounds, exert immunosuppressive effects, reduce the pathological production of proinflammatory cytokines driven by JAK-

STAT signaling, and inhibit gain-of-function JAK mutants (185,186). Figure 1.3 summarizes the principal types of drugs targeting the JAK-STAT signaling pathway (187).

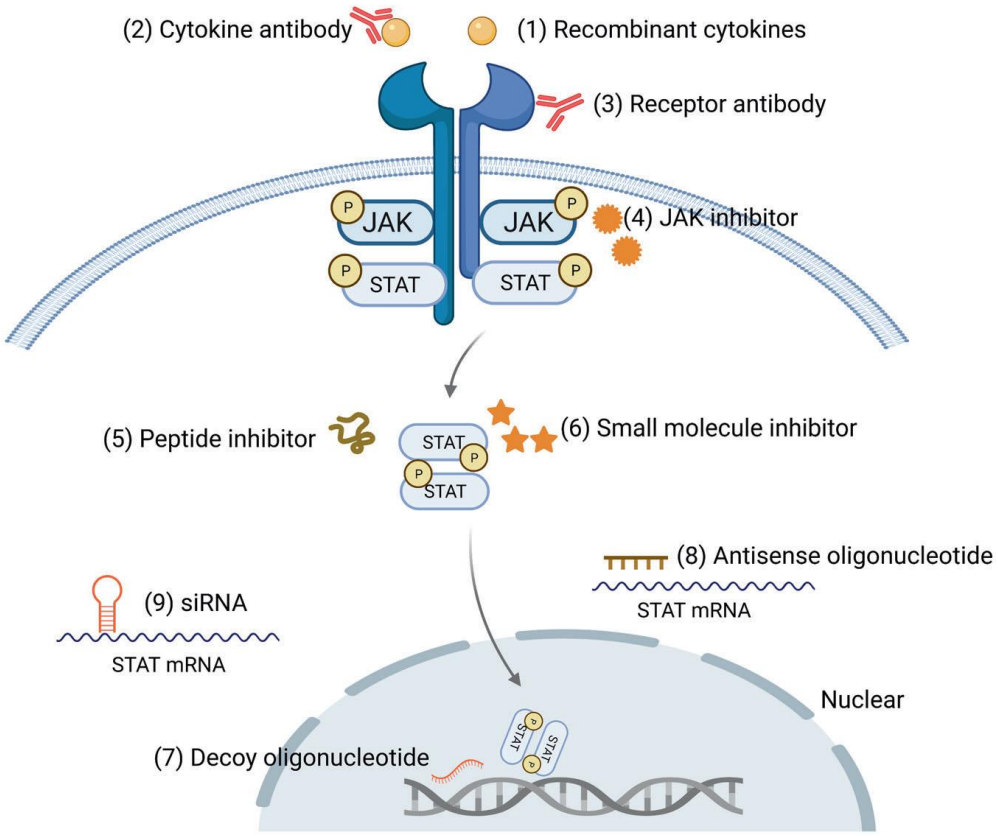

***Figure 1.3. Therapeutic targets of the JAK-STAT signalling pathway (from** (187)*
(1) Recombinant cytokines, (2) cytokine antibodies, and (3) receptor antibodies are designed to target cytokines or receptors; (4) JAK inhibitors are designed to target JAKs; and (5) peptide inhibitors, (6) small-molecule inhibitors, (7) decoy oligonucleotides (ODNs), (8) antisense oligonucleotides (ASOs), and (9) siRNAs target STAT.

Given its extensive use in the treatment of UC treatment and the lack of information on the remaining target cells, in this thesis we will focus on the first-generation JAK inhibitor tofacitinib.

Tofacitinib, also known as Xeljanz or CP690550, is a small molecule and, along with baricitinib, was the first orally available JAK inhibitor approved for the treatment of rheumatoid arthritis, psoriasis and ulcerative colitis (188). Tofacitinib has demonstrated efficacy in inducing and maintaining remission in UC (189), and in reducing the disease severity in rheumatoid arthritis patients (190). It primarily inhibits JAK1 and JAK3, with a lesser effect on JAK2 and TYK2. Tofacitinib acts by blocking the Ɣc cytokine-receptor signaling pathway via JAK1 and JAK3 in T cells, thereby disrupting Th1 and Th2 differentiation and reducing the production of inflammatory Th17 cells. Additionally, it

suppresses cytokine production in both innate and adaptive immune responses, targeting key cytokines such as IFN-γ, TNF, IL-6, IL-12, IL-17, and IL-23 (191).

Multiple studies have demonstrated the ability of tofacitinib to shift MΦ polarization toward an anti-inflammatory M2-like phenotype in human and murine bone marrow-derived macrophages (BMDM) (192), while also modulating M1 macrophages derived from human peripheral blood monocytes, reducing IL-6 production and increasing IL-1β, IL-23, and IL-10 levels (193). Notably, tofacitinib has been also reported to impair M2-like MΦ development under certain conditions by downregulating CD206 expression and IL-10 production in monocyte-derived macrophages (MO-MΦs), thereby reducing the development of the M2 macrophage phenotype (193). Additionally, it suppresses the expression of immunostimulatory surface molecules such as CD80, CD86, CD83, and CD40 in both M1- and M2-polarized human MO-MΦs, thereby reducing their activation potential (193). While these findings are remarkable, it is important to highlight that M1 and M2 polarization models (induced with LPS/IFNγ and IL-4, respectively) are only methodological approximations, as MΦs exist along a continuum of phenotypes rather than discrete M1 or M2 states. Regarding T cell function, tofacitinib has been shown to significantly suppress the proliferation and differentiation of peripheral blood Th1 and Th17 cells, key drivers of inflammation in IBD (194). This effect is likely mediated by JAK1 and JAK3 inhibition, leading to reduced production of inflammatory cytokines such as TNF-α, IL-6, IL-17, and IFN-γ in human peripheral blood $CD4^+$ T cells (190,195)

The effect of tofacitinib on DCs has been less extensively studied. However, research using monocyte-derived dendritic cells (MO-DCs) and bone marrow-derived dendritic cells (BMDCs) has provided some insights. In MO-DCs, tofacitinib did not suppress MHC-II expression, but it downregulated CD80/CD86 expression as well as the transcription factor IRF7, which promotes type I IFN production (196). Additionally, in human MO-MO-DCs, tofacitinib was shown to suppress their T cell stimulatory capacity, further modulating the adaptive immunity (196). In contrast, tofacitinib-treated BMDCs displayed a strongly impaired ability to polarize $IFNγ^+$ T cells, while promoting an increase in $IL\text{-}17^+$ T cells and a slight increase in $FOXP3^+$ regulatory T cells (192)

Summarizing, Tofacitinib emerges as a potent immunomodulator with broad applications across inflammatory and autoimmune diseases. Its primary mechanism involves JAK1/JAK3 inhibition, leading to a reduced inflammatory cytokine production in human and murine T cells, modulation of human MO-MΦ polarization, suppression of DC activation, and restoration of immune balance through Treg promotion and Th1/Th17 inhibition (194). While its ability to skew immune responses toward a regulatory state makes it highly

effective, some paradoxical effects—such as impaired M2 macrophage polarization and suppression of antimicrobial peptides—highlight the need for further research to optimize its therapeutic use and minimize potential adverse effects (193,197).

Last, but not least, it is important to note that most studies analysing tofacitinib's effects on MΦs and DCs use *in vitro* models, such as BMDMs, MO-MΦs, and MO-DCs. However, no studies have directly examined human intestinal MΦs or DCs isolated from the gut (i.e. *bona fide* DC and MΦ). While bone marrow-derived and monocyte-derived cells provide valuable experimental models, they do not fully replicate the phenotype and function of tissue-resident intestinal immune cells (198,199). These *in vitro*-derived cells lack exposure to the intestinal microenvironment, which plays a critical role in conditioning their activation state, cytokine profile, and functional properties. Consequently, while these studies provide significant insights into the immunomodulatory effects of tofacitinib, caution is required when extrapolating these findings to “real” intestinal MΦs and DCs. Future research should focus on assessing tofacitinib’s effects on primary intestinal immune cells to improve our understanding of its precise mechanisms of action in IBD.

1.5 GUT MICROBIOTA IN IBD: BACTERIAL AND FUNGAL INTERACTIONS

1.5.1 Composition and function of a healthy gut microbiota

The GI mucosal system serves as the primary point of direct interaction with external antigens, potentially associated with the necessity of managing the diverse and dynamic populations of commensals. Besides, although the GI mucosal system operates separately from the broader immune system, it is inseparable from the systemic immune system. The GI-tract contains a variety of bacteria (bacteriome), which comprise most of it, as well as archaea, fungi (mycobiome) and virus (virome), collectively termed the “gut commensal microbiome”. Commensals have formed a relationship of mutually beneficial coexistence with the host. Indeed, the microbiota that live in the GI-tract help the host in many ways such as metabolic, trophic, immunologic and intestinal defense functions (200) while commensals take advantage of the host by breaking down dietary fibers and other food metabolites that the host cannot digest on its own, producing short-chain fatty acids (SFCA) and other metabolites that can be absorbed and utilized by the host (200). The GI-tract harbours between 10^{13} and 10^{14} microorganisms, in which about 3.8×10^{13} are bacterial cell -same number as human cells, belonging to about 1000 different species primarily comprised of members of phyla *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Proteobacteria* (201,202). As a result, our mucosal surfaces interact closely with the resident microbiota, forming a "supraorganism"—a concept that encompasses both the host and the diverse microbial communities residing within it (200). However, microbial communities show high diversity between individuals (203), which makes it challenging to define what constitutes a balanced microbial community in a healthy state.

Each mucosal surface is composed of distinct cell types and generates unique microenvironments, shaping the biogeography of the microbiota. The distribution of microbial communities along the gastrointestinal tract is influenced by gradients of nutrients, chemical factors, and localized immune responses. For example, the small intestine has a more acidic environment, higher oxygen levels, and an increased presence of antimicrobial compounds. Consequently, the bacteriome of the small intestine is primarily composed of rapidly growing facultative anaerobes (204) belonging to the class *Bacilli* (phylum *Firmicutes*) and the order *Enterobacterales*, whereas obligately anaerobic primary fermenters belonging to the classes *Bacteroidia* (phylum *Bacteroidetes*) and *Clostridia* (phylum *Firmicutes*) dominate the microbial community in the large intestine (205).

Diet also shapes the gut microbiota composition during homeostasis (205). Under normal homeostatic conditions, the host regulates oxygen and nitrate concentrations at stable levels throughout different sections of the intestine (206,207). However, fluctuations in the

availability of diet-derived electron donors can lead to changes in microbiota composition. In the GI-tract, food is broken down by host enzymes in the upper digestive system into simple sugars, amino acids, fatty acids, and diglycerides. These nutrients are efficiently absorbed in the small intestine where they serve as major electron donors for the colonic microbiota. In contrast, indigestible polymers—such as plant polysaccharides (fiber) and cartilage-derived glycans—act as diet-derived electron donors available to colonic microbiota (208). Another category of diet-derived electron donors in the colon consists of simple sugars that are poorly absorbed in the small intestine (fermentable oligosaccharides, disaccharides, monosaccharides, and polyols, FODMAPs) (209). During homeostasis, the host restricts the availability of exogenous electron acceptors such as oxygen and nitrate in the colonic lumen. As a result, FODMAPs and indigestible polymers are metabolized by obligate anaerobic primary fermenters using endogenous electron acceptors. The ATP generated through these redox reactions supports the dominance of obligate anaerobic primary fermenters within the colonic microbiota under homeostatic conditions (205). In this way, microorganisms increase in both concentration and complexity as they migrate through the GI-tract (204).

On the other hand, fungi constitute around 0.1% of the gut microbiome and engage in both antagonistic and/or synergistic interactions with bacteria and viruses within the gut (210). The fungal population gradually increases along the intestinal tract, from the ileum to the colon, being at the highest concentration in the distal colon (210). The diversity and abundance of fungi in the GI-tract are significantly lower than those of bacteria, and their composition is considered heterogeneous and relatively unstable (211). The human intestinal mycobiome is primarily composed of three major phyla: *Ascomycota*, *Basidiomycota*, and *Chytridiomycota.* It seems that only few genera are the main components of the gut mycobiome. *Candida spp.*, *Penicillium commune*, *Saccharomyces cerevisiae*, *Aspergillus versicolor*, *Cryptococcus spp.*, *Malassezia spp.*, *Cladosporium herbarum*, *Galactomyces geotrichum*, *Debaryomyces hansenii* and *Trichosporonspp*. have been frequently reported from the gut (212), while other fungal genera, including *Aspergillus*, *Cryptococcus*, *Rhodotorula*, *Mucor*, and *Trichosporon*, are occasionally detected (210). Fungal composition also depends on several factors as the diet, and subsequently there exists cultural and regional differences in the composition of the intestinal mycobiota. For example, the mycobiome of healthy Japanese mainly consists of the phyla *Ascomycota* and *Basidiomycota* (213), similarly as the mycobiome from Western populations (214), however there are differences at the genus level. The genus *Saccharomyces* is dominant in both Japanese and Western populations, but other major taxa of the Japanese population, i.e., the genera *Sarocladium* and *Leucosporidium*, were

not detected in Western population (213,214). In contrast, major taxa reported in Western populations, i.e., the genera *Debaryomyces* and *Penicillium*, were not detected in the Japanese samples (214).

Fungi can respond to diverse environmental conditions of the GI-tract through metabolic shifts, as bacteria (215), and as a unique characteristic of certain fungi, adopting multiple morphological growth forms (216,217). This unique trait adds an extra layer of complexity to host-mycobiome interactions.

Last, the gut virome is challenging to study due to the difficulty of isolating their DNA without contamination from human DNA and consequently, the low number of reads. Despite this, most studies show that the human gut virome is predominantly composed of bacteriophages (phages), which plays a crucial role in maintaining gut homeostasis and influencing pathogenic conditions through its interactions with the gut bacterial community (218,219). Viruses that infect prokaryotic cells (bacteria and/or archaea) constitute approximately 90% of all viruses, while the remaining 10% are eukaryotic viruses that infect plants and animals, including humans (220). Phages replicate and proliferate within infected bacterial cells and are subsequently released through cell lysis in the lytic cycle (220,221). This lytic process alters the composition of bacterial populations and significantly contributes to shaping the gut microbiota. In the gut, many phages exist in a lysogenic or latent state, persisting as integrated prophages within their bacterial hosts (218). This integration can modify bacterial immunogenicity, thereby influencing bacteria-host interactions, as well as bacterial functions such as antibiotic resistance and toxin production (219,220). In healthy individuals, the human gut virome exhibits high interindividual diversity while remaining temporally stable (222,223). Among healthy individuals, phages belonging to the order *Caudovirales* or the family *Microviridae* are predominant, primarily existing in a latent state within their bacterial hosts and producing limited viral progeny capable of infecting and lysing other bacteria (222,224).

1.5.2 Importance of microbiota on human health: role of microbial metabolites in immune modulation

The microbiome collaborates with the host to establish a host-microbiota co-metabolism system to contribute to various metabolic processes in the human body. Complex carbohydrates can be fermented by bacteria, generating metabolites such as SCFAs, which serve as crucial chemical mediators between the microbiota and the host. It is suggested that SCFAs have combined effects that support intestinal, hepatic, and overall glucose homeostasis (225). Additionally, microbial metabolism plays a role in bile acid,

choline and tryptophan metabolisms, as well as other biochemical pathways (204). By enhancing the expression of intestinal nutrient transporters, gut microbes facilitate nutrient supply to the host (226).

One key process that microbiota supports is the fermentation of undigested or partially digested dietary fibers in the colon, which generates SCFAs such as butyric acid, propionic acid, and acetic acid. While fungi produce higher amounts of methane, acetate and formate, bacteria produce more butyrate and propionate (227). These SCFAs can pass through the intestinal epithelium and interact with host cells, thereby influencing immune function (228) as well as serve as crucial energy sources not only for the gut microbiota but also for IECs (229) or colonocytes (230). SCFAs further stimulate intestinal gluconeogenesis, aiding in the production of supportive lipids (231), and enhance epithelial barrier integrity and promoting immune tolerance through various mechanisms: i) increasing mucus production by intestinal goblet cells (232); ii) inhibiting the NF-κB signaling pathway, which reduces inflammation (233); iii) activating inflammasomes, leading to the production of IL-18 (234); iv) stimulating B cells to secrete secretory IgA (sIgA), which helps regulate the gut microbiota (235); v) reducing the expression of T cell-activating molecules on APCs (236), vi) increasing the number of Tregs in the colon, along with their expression of FOXP3 (237) and vii) production of anti-inflammatory cytokines like IL-10 (238). Moreover, the gastrointestinal microbiota participates in the *de novo* synthesis of essential vitamins that the host cannot produce, such as Vitamin B12 (239). Another way the microbiota influences the host is by inducing epigenetic changes in host cells. SCFAs and other microbial metabolites regulate histone acetylation, which can modify gene expression. A notable example is butyrate's role in promoting Treg cell differentiation through epigenetic mechanisms, demonstrating the microbiota's impact on immune regulation (240). Additionally, the microbiota can alter DNA or histone methylation, further influencing immune responses (241,242).

Bile acids (BAs) are host-derived metabolites synthesized from cholesterol in the liver. Primary BAs, cholic acid and chenodeoxycholic acid, are produced through a multi-enzyme process and conjugated with either taurine or glycine. These amphipathic molecules play a crucial role in facilitating lipid digestion and absorption in the small intestine. In the colon, primary BAs undergo microbiota-driven deconjugation, where the amino acid moiety is removed, converting them into secondary BAs. These transformations are mediated by specific bacterial taxa, including *Bifidobacterium, Lactobacillus, Bacteroides, Clostridium, Eubacterium*, and *Listeria* (243). BAs have an anti-inflammatory role as they promote RORγ^{+} Treg cells via BA-vitamin D receptor signaling (244).

Tryptophan is an essential amino acid obtained through diet, with the GI-tract serving as the primary site of its metabolism. This process generates several bioactive compounds, including serotonin, vitamin B3, melatonin, and various metabolically significant intermediates such as anthranilates, indoles, kynurenates, and quinolines, each with diverse biological functions. Tryptophan can be metabolized by the host (245) or by gut microbiota, which converts tryptophan into indole-based compounds. Increased tryptophan metabolism, along with a reduction in indole-derived metabolites, which serve as aryl hydrocarbon receptor (AhR) ligands, has been strongly implicated in IBD pathogenesis (246). A limited number of bacterial taxa, including *Peptostreptococcus russellii*, *Clostridium sporogenes*, and members of the *Lactobacillus* genus, have been identified as producers of these indole-based metabolites. These compounds act as key agonists of AhR, triggering the transcriptional activation of genes involved in the anti-inflammatory response in T cells and innate lymphoid cells (247).

1.5.3 Dysbiosis in IBD: shifts in bacterial and fungal populations

Gut dysbiosis, which is characterized by the loss of homeostatic balance of beneficial and detrimental microorganisms in the gut, is a characteristic feature of IBD, and occurs through the disruption of the intestinal barrier and changes in the intestinal microbiome (248). However, whether these microbial alterations are the cause, or an effect of the IBD-associated inflammation stays elusive.

Gut microbial dysbiosis in patients with IBD has been characterized by reduction in diversity and loss of beneficial gut microbial genera and by colonization of potential pathobionts. IBD patients have reduced bacterial biodiversity, with a a consistent reduction in beneficial bacterial taxa such as *Faecalibacterium prausnitzii* and *Roseburia hominis*, along with an increase in facultative anaerobes, including members of the *Enterobacteriaceae* family and known inflammation-associated species like *Ruminococcus gnavus*. Both CD and UC samples exhibit decreased gut microbiome stability, as evidenced by the loss of previously prevalent microbial members and the emergence of new taxa over time. These microbial changes are also associated with reduced fecal SCFA levels, as well as elevated concentrations of primary BAs (cholate) and acyl-carnitines, further highlighting the metabolic shifts linked to IBD-related dysbiosis (249). The overgrowth of harmful bacterial species has been recognized for its pro-inflammatory effects, contributing to the pathogenesis of IBD. Pathobionts such as adherent-invasive *Escherichia coli*, enterotoxigenic *Bacteroides fragilis*, *Campylobacter concisus*, *Fusobacterium nucleatum*,

and *Mycobacterium avium* subsp. *paratuberculosis* have been reported to be elevated in human IBD studies (248).

Adherent-invasive *Escherichia coli* (AIEC) has been strongly linked to CD as it adheres to abnormally expressed CEACAM6 (a glycoprotein expressed in IECs) in the intestinal epithelium and invades the lamina propria, triggering an inflammatory response. AIEC persistence is facilitated by impaired autophagy in MΦs, leading to increased production of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-8. Additionally, its metabolism of 1,2-propanediol has been associated with Th17 cell induction and IL-1β production (250,251). Enterotoxigenic *Bacteroides fragilis* (ETBF) has been linked to UC, colitis-associated cancer, and colorectal cancer due to its *B. fragilis* toxin, which activates the Th17 inflammatory pathway (252). Similarly, the oral bacterium *Campylobacter concisus* has been associated with an increased IBD risk, especially when carrying the enteropathogenic Zot virulence protein, which disrupts epithelial tight junctions and enhances immune sensitivity to commensal flora (253). *Fusobacterium nucleatum*, found in higher abundance in IBD tissues, promotes inflammation through autophagic epithelial cell death, M1 macrophage polarization, and Th1/Th17 differentiation, leading to increased TNF-α, IFNγ, IL-1β, IL-6, and IL-17 levels. Additionally, *F. nucleatum* is linked to colorectal cancer progression via activation of the TLR4-Myd88 and NF-κB signaling pathways (254,255). *Eggerthella lenta*, another bacterium enriched in IBD patients, has been shown to promote a Th17 response in mice (256). IBD is also characterized by an increase in hydrogen sulfide-producing bacteria, such as *Desulfovibrio* and *Bilophila wadsworthensis*. These sulfate-reducing bacteria contribute to gut toxicity by producing H_2S while also depleting beneficial *Firmicutes* of essential carbon sources. *B. wadsworthensis* has been associated with Th1-mediated colitis in IL-$10^{-/-}$ mice, while *Desulfovibrio* has demonstrated to exacerbate colitis (257,258). Sulfide-producing bacteria are particularly abundant in inflamed pouches of UC patients (a complication of UC), where they metabolize sulfomucin, reducing butyrate availability and contributing to epithelial damage. Finally, other pathobionts such as *Enterobacteriaceae, Fusobacterium spp., Clostridium perfringens, Escherichia coli,* and *Clostridium difficile* have been associated with pouchitis, further emphasizing the role of dysbiosis in IBD pathogenesis (259,260).

While pathobionts increase, there is also a loss of beneficial bacteria in IBD. IBD involves a decline in the abundances of beneficial gut bacteria that are active SCFA producers such as *Faecalibacterium prausnitzii* and species of *Roseburia*, *Eubacterium*, *Dorea*, *Blautia*, *Holdemanella* and other members of *Firmicutes* and *Bacteroidetes phyla* (261). Both CD and UC are characterized by a reduction in secondary BA levels and an increase in the primary BA pool within the gut. Similarly, pouchitis exhibits lower concentrations of both

secondary BAs and SCFAs. This reduction is likely attributed to inflammation-driven decreases in the abundances of bacterial taxa belonging to the *Lachnospiraceae* and *Ruminococcaceae* families (262). Metabolomic screens in patients with IBD have highlighted reduced secondary BA pool and elevated primary BAs, which could be driven by impaired microbiota mediated deconjugation, transformation and desulfation of primary BAs (244).

The fungal composition also changes in IBD. Some studies have shown that fungal diversity increases in CD patients (263), while decreases in UC (214). A study of the faecal mycobiome from IBD patients and controls showed that fungal dysbiosis in IBD is marked by a shift in the ratio of dominant fungal phyla, particularly an increased *Basidiomycota/Ascomycota* ratio (214). The abundance of *Saccharomyces cerevisiae* is significantly reduced in IBD patients, while *Candida albicans*, a known opportunistic pathogen, is elevated, particularly in CD. Additionally, *Malassezia* species, are more abundant in the gut of IBD patients, suggesting a potential role in intestinal inflammation (214). Other works that study mucosal mycobiome observed that, as occurs in faeces, the colonic mucosa-associated fungal microbiota was dominated by *Basidiomycota* and *Ascomycota* phyla (264). *Cystofilobasidiaceae* family and *Candida glabrata* species were overrepresented in CD patients. *Saccharomyces cerevisiae* and *Filobasidium uniguttulatum* species were associated with non-inflamed mucosa, whereas *Xylariales* order was associated with inflamed mucosa (264).

The specific IBD phenotypes are linked to distinct mycobiome profiles. In CD, the mycobiome composition varies depending on whether the disease affects the ileum or is confined to the colon. Notably, CD cases sparing the ileum exhibited higher levels of *Candida* and *Debaryomyces*, whereas *Aspergillus* and *Pichia* were more abundant in ileal-involved CD (265). During disease flares, the *Basidiomycota/Ascomycota* ratio increases compared to healthy controls (265). At the species level, CD patients with active flares showed a greater relative abundance of *Candida* species, *Gibberella moniliformis*, *Alternaria brassicicola*, and *Cryptococcus neoformans* (266). In UC, mycobiome variations were most evident when categorized by disease extent. Patients with proctitis displayed the highest relative abundance of *Penicillium*, which negatively correlated with the proximal progression of the disease (265). In contrast, *Pichia* was overrepresented in patients with left-sided colitis compared to those with proctitis (265).

These functional alterations are reflected in the disease-associated remodeling of the gut metabolome. Given the critical role of gut dysbiosis in IBD pathogenesis, therapeutic strategies aimed at microbiome restoration, such as fecal microbiota transplantation, have

demonstrated both efficacy and safety in inducing and maintaining remission in UC patients (267,268).

1.6 Overview

To sum up, cDCs are pivotal orchestrators of intestinal immune responses, acting as a bridge between innate and adaptive immunity through their capacity to sample antigens and shape T cell differentiation. In the gut, cDCs play essential roles in maintaining mucosal tolerance and responding to microbial and dietary antigens. However, in the context of IBD, the function and phenotype of cDC are altered, contributing to the loss of immune homeostasis and the amplification of pro-inflammatory responses. Despite their known importance, most current insights into DC biology in IBD arise from murine models or monocyte-derived DCs, which may not accurately reflect the dynamics of human intestinal DCs. Moreover, dysregulated microbial communities in IBD further modulate immune function, promoting aberrant immune overactivation. Therefore, this thesis will focus on characterizing the phenotypic and functional properties of human intestinal cDC in health and IBD context, assessing the immunomodulatory effect of tofacitinib on these cells, and exploring the interplay between microbial dysbiosis and immune responses.

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Chapter 2. Objectives

1. To optimize a standardized protocol for the isolation of human intestinal lamina propria mononuclear cells (LPMCs), enabling the isolation and experimental use of dendritic cells (DCs), monocytes, and macrophages from human intestinal tissue.
2. To characterize human intestinal conventional DC (cDC) in both healthy individuals and patients with inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), during active status of the diseases and remission phases.
3. To investigate the function of intestinal cDCs in health and IBD, assessing how their source (control or IBD mucosa) and phenotype affect their stimulatory capacity over naïve T cells.
4. To perform an unsupervised analysis of human intestinal antigen-presenting cells (APCs), using dimensionality reduction and clustering algorithms to perform deeper characterization analysis.
5. To study the immunomodulatory effects of tofacitinib on intestinal APCs from control patients under basal conditions and upon inflammatory stimulation with lipopolysaccharide (LPS).
6. To assess the effects of tofacitinib on APCs from patients with UC (both active and quiescent patients).
7. To evaluate how tofacitinib modulates the function of human intestinal cDCs.
8. To characterize the intestinal microbiota—including bacteria, fungi, and viruses—in human intestinal biopsies from both healthy controls and IBD patients (CD and UC, in both active and remission states).
9. To analyze the bacterial composition (bacteriome) of faecal samples, comparing healthy and IBD cohorts.
10. To compare the bacterial genera present in intestinal mucosa and faeces, identifying similarities and differences across control subjects and IBD subtypes (active/quiescent UC and active/quiescent CD).
11. To explore correlations between fungal and bacterial communities in the intestinal mucosa of both control and IBD patients.
12. To identify specific bacterial genera that could serve as potential fecal biomarkers for IBD diagnosis or disease monitoring.

Chapter 3. Materials and methods

3.1 PATIENTS AND BIOLOGICAL SAMPLES

Samples from six different cohorts of patients were used through this thesis, including: healthy controls (HC), Crohn's disease (CD) patients, differentiating between endoscopically active Crohn (aCD) and quiescent Crohn (qCD); ulcerative colitis (UC), both endoscopically active (aUC) and quiescent (qUC) and patients with colorectar cancer (CRC).

Biopsies and blood samples were taken during the normal course of a gastroscopy or colonoscopy at the gastroenterology service from either Hospital La Princesa (Madrid, Spain), or Hospital Clínico Universitario or Hospital Universitario Río Hortega, both from Valladolid (Spain) in the context of a routinary endoscopy or colonoscopy for the diagnosis and monitoring of the disease. In the case of control individuals, they had been referred due to rectal bleeding, dyspepsia or colorectal cancer screening, but in all cases they had macroscopically and histologically normal mucosa. The location of the biopsies, SES-CD, UCEIS, and Mayo Endoscopic scores, patients' gender and age, as well as other relevant data for the study, are detailed in the specific chapters.

Stool samples were also obtained during the 24 hours prior to the colonoscopy.

Ileal and colonic resection were obtained from the proximal and distal ends from patients with colorectal cancer, with a minimum distance of 10 cm from the tumors (used as control tissue) and, in a similar manner, the affected and non-affected tissue from CD patients subjected to tissue resection were also obtained.

Peripheral blood samples were also obtained from healthy controls provided by the "Biobanco del Centro de Hemoterapia y Hemodonación de Castilla y León" (Valladolid, Spain).

In all cases, samples were obtained following written informed consent after ethical approval from the local ethics committee from Hospital La Princesa (Madrid, Spain), Hospital Clínico Universitario (Valladolid, Spain) and Hospital Rio Hortega (Valladolid, Spain) (PI 19-1353, PI-19-1430 and PI 22-2869). To ensure patient confidentiality, all samples were assigned unique identifiers that do not reveal any personal information using the coding system for later analysis.

Table 3.1 summarizes the number of samples from each cohort of study that had been used in all this work.

Table 3.1. Number of patients of the different cohorts that had been used throughout the present work.

Cohort	Number of samples		
	cDC studies (Chapter 5)	**Tofacitinib studies (Chapter 6)**	**Microbiome studies (Chapter 7)**
Healthy controls	61	10	5
Active Crohn's Disease	12	-	6
Quiescent Crohn's disease	7	-	6
Active colitis	15	10	5
Quiescent colitis	11	10	7
Colorectal Cancer	7	6	-
Crohn's Disease subjected to resection	6	-	-

3.2 SAMPLE COLLECTING AND PROCESSING

3.2.1 Blood processing

Peripheral blood mononuclear cells (PBMC) were obtained from buffy coats from healthy donors as well as and from blood samples from healthy mucosal controls and IBD patients (aCD, qCD, aUC and qUC), collected in Heparin-Lityhium tubes (BD Vacuatiner® cat # 367526). Concentrated blood from buffy coats were first diluted in Phosphate-buffered saline (PBS) 1:2 (Cytiva, cat # SH30028.02).

In both cases (whole blood and diluted blood from buffy coats) were processed by centrifugation over Ficoll-Paque PLUS (Cytiva, cat # 17144003). First, 3 mL of Ficoll-Paque Plus was added to a 15 mL tube (Corning™, cat # 10579691), ensuring it was not adhered to the tube walls. Next, a maximum of 9 mL of diluted blood was carefully layered on top of the Ficoll solution using a sterile Pasteur pipette (Fisherbrand™, cat # 13439108), ensuring minimal mixing. The tube was then centrifuged at 800g for 30 minutes at 4°C and no brake, allowing the separation of blood components based on density. Following centrifugation, the PBMC layer, appearing as a distinct white band between the plasma and Ficoll, was carefully collected and transferred into a new tube containing 3 mL of RPMI (Gibco, cat # 11875093). To remove residual Ficoll and plasma, isolated PBMCs were washed by adding RPMI up to 8 mL, followed by centrifugation at 300g for 5 minutes at 4ºC. Resulting pellet was then resuspended in 2 mL of fresh RPMI, ensuring cell viability in case of downstream cell culture or in PBS containing 1 mM Ethylenediaminetetraacetic acid (EDTA) (Invitrogen, cat # 15575-038) and 0.02% sodium azide (Sigma-Aldrich, cat # S2002-25G) (FACS buffer) in case of downstream flow cytometry staining.

3.2.1.1 Naïve T cell enrichment

T cell enrichment was first carried out using magnetic beads and following manufacturer's instructions of Pan T Cell Isolation Kit human (Miltenyi Biotec, cat # 130-096-535), which capture cells thanks to their specificity. Labelled cells were passed through an LS column (Miltenyi Biotec, cat #·130-042-401) coupled to a magnet, and only those cells that were not of interest were retained on the mesh of the column, previously conditioned with MACS buffer, which consists in PBS containing 2 mM EDTA + 0,5% Bovine Serum Albumine, (BSA) (Gibco, cat # 30063-572). Therefore, isolated T cells were those that eluted from the column.

From those T cells, naïve T cells were obtained. Starting from enriched T lymphocytes, the REAlease® CD62L MicroBead kit (Miltenyi Biotec, cat # 130-124-203) was used following manufacturer's instructions. This kit retained $CD62L^+$ cells in the magnetic LS column and therefore a specific buffer provided in the kit was used to allow CD62L+ cells to be released.

3.2.2 Intestinal biopsies

In all cases, biopsies were collected immediately following extraction in a maximum of 30 minutes in ice-chilled RPMI (Gibco, cat # 11875093) at 4ºC and then transported to the lab maintaining cold conditions (4ºC).

For the obtention of lamina propria mononuclear cells (LPMC), biopsies were first incubated in 5 mL of HBSS (Gibco, cat # 24020117) supplemented with 1 mM dithiothreitol (DTT) (Sigma-Aldrich, cat # 43816) and 1 mM ethylenediaminetetraacetic acid (EDTA) (Invitrogen, cat # 11568896) on an orbital shaker (30 minutes, 250 rpm, 37°C). After incubation, the supernatant was discarded, and the tissue was subjected to a second incubation under identical conditions to remove the mucus layer, enterocytes, and intraepithelial leukocytes. Subsequently, the remaining tissue was enzymatically digested in 5 mL of RPMI medium containing 1 mg/mL collagenase D (Roche, cat # 11088882001), 20 µg/mL Liberase™ TL Research Grade (Roche, cat # 5401020001), and 25 U/mL benzonase (Universal Nuclease for Cell Lysis, Pierce™, ThermoFisher Scientific, cat # 88702) to obtain finally LPMC. The digestion was performed on an orbital shaker (MaxQ 4450, TermoFisher, cat # SHKE4450) (three incubations, 30 minutes each, 250 rpm, 37°C). After each incubation, the medium was filtered through a 100 µm strainer (Fisherbrand™, cat # 22363549) into a 50ml tube (Corning™, cat # 10334131) to isolate LPMC, which were maintained at 4°C until finishing all the process. The remaining tissue underwent two additional digestion cycles following the same protocol. After all incubations, the collected LPMC were pooled into a single 50 ml tube, centrifuged (300g, 10 min, 4°C), and resuspended in RPMI for immediate use (see 3.4 or 3.3.1 sections) or cryopreservation. For cryopreservation, the cells were suspended in freezing medium composed of RPMI supplemented with Foetal Calf Serum (FCS) (Gibco, cat # 10500064) and 10% Dimethyl Sulfoxide (DMSO) (MP Biomedicals, cat # 190186) until needed. Cryopreservation was performed in cryogenic vials (Fisherbrand™, cat # 300460-0020), which were stored for at least 24 hours in a CoolCell™ cell freezing container (Corning™, cat # 432001) to ensure a controlled freezing rate.

For microbiome studies, biopsies were preserved in PBS (Cytiva, cat # SH30028.02) at 4°C just after being obtained and then, cryopreserved in RNAlater™ (ThermoFisher Scientific, cat # AM721) at -80°C in cryogenic vials (Fisherbrand™, cat # 300460-0020) until being sent in cold conditions to sequencing.

For cytokine analysis, one biopsy per patient was cultured overnight in 0,5 ml of RPMI medium (Gibco, cat # 11875093) supplemented with 100 U/ml Penicillin-Streptomycin (Gibco, cat # 11548876) and 60 µg/ml Gentamicin (Gibco, cat #11520506) in 24 flat-bottom

plates (TermoFisher Scientific, cat # 142475) at 37°C and 5% CO_2. After 18h culture media was centrifugated at 400g for 5 min, pellet was discarded, and the cell-free supernatant was cryopreserved in cryogenic vials (Fisherbrand™, cat # 300460-0020) at -80°C until use.

3.2.3 Intestinal resection

Human intestinal resections were collected immediately following surgery in ice-chilled RPMI medium (Gibco, cat # 11875093) (4ºC) and then transported to the lab maintaining cold conditions (4ºC). Tissue was cleaned with HBSS, and muscle and fat were removed using surgical scissors. When tissue was clean, it was cut into pieces of about 1 cm^2 in order to process them separately in 15 ml tubes (Corning™, cat # 10579691). At this point, tissue was processed exactly as biopsies following chemical and enzymatic digestions as explained above (see “Intestinal biopsies section”).

3.2.4 Fecal samples

Stool samples were collected in fecal collection tubes (Canvax Biotech, cat # SC0012) during the 24 hours prior to the colonoscopy and then, frozen at -80°C until use.

3.3 FLOW CYTOMETRY APPROACHES

3.3.1 Antibody Staining

Staining of the isolated cells (both PMBC or LPMC) was carried out in polystyrene tubes (Falcon™, cat # 352054). First, a viability dye, Live/Dead™ Near-IR (Invitrogen, cat # 10154363), was added and incubated for less than 1 minute at room temperature (RT), and then, washed in FACS buffer at 400g for 5 minutes. After that, nonspecific binding was blocked using Fc-block (BD Pharmingen, cat # 564220) for 10 minutes at RT. Cells were subsequently washed in FACS buffer at 400g for 5 minutes. Extracellular staining was then incubated for 20 minutes at 4°C. For intracellular staining (if required), the Fix and Perm kit (Invitrogen, cat # GAS004) was employed following the manufacturer's protocol. In brief, cells were fixed with Medium A for 15 minutes at RT. After a FACS-washing step, cells were permeabilized with Medium B and incubated with the primary intracellular antibody for 20 minutes at RT and then washed again (centrifuging at 400g, 5 min). In all procedures, the cells were fixed using Fixing Medium consisting in PBS (Lonza, cat # 17-516F) with 1% Buffered Formalin (Protocol, cat # 032-059), for 10 minutes at 4°C. After washing in FACS buffer, the cells were acquired within 48 hours using a flow cytometer (Gallios Beckman Coulter or Aurora Cytek 5 lasers).

3.3.2 Supervised analysis

Supervised data analysis was performed with the FlowJo software (BD Biosciences) and on the OMIQ Data Science platform (©Omiq, Inc. 2024), depending on the origin of the data (Gallios Beckman Coulter flow cytometer or Aurora Cytek spectral flow cytometer, respectively). In the spectral cytometer files, first step was to transform the fluorescence data using the scale parameters suggested by the software. Subsequently, a cleaning algorithm (PeacoQC) was run on all samples. Standard flow cytometer data was compensated before acquisition, so they did not need a subsequently fluorescence data transformation in FlowJo. In both analysis software (FlowJo and OMIQ) cells were identified by following hierarchical gating strategies that enabled to distinguish different immune cells (i.e. dendritic cells, macrophages, etc) and different cell markers (TNFα, IL10, IL6, CXCR3…). In all cases, gating strategies started discarding doublets -comparting FSC-A and FSC-H- and selecting total viable leukocytes ($CD45^+$ cells that were negative for the viability dye) and followed with the specific gates depending on the cells of interest.

Fluorescence Minus One (FMO) method was used to define positive populations in all cytometry panels. Briefly, in an FMO control, all fluorochromes in a given panel were included except for the one being evaluated, allowing to account for spectral spillover and

autofluorescence. This approach helps to distinguish true positive signals from background noise and compensatory artifacts, ensuring precise data interpretation.

3.3.3 Cell sorting

LPMC obtained from tissue resections were used to enrich cDC1, cDC2, cDC2 $CD103^+$ cDC subsets as well as MΦ by flow cytometry sorting. In this way, total LPMC were stained using first a viability dye, Live/Dead™ Near-IR (Invitrogen, cat # 10154363), incubated for 1 minute at RT, followed by a wash step in FACS buffer. Then, a nonspecific binding blocker, Fc-block (BD Pharmingen, cat # 564220) was incubated for 10 minutes at room temperature (RT). Cells were subsequently washed in FACS buffer at 400g for 5 minutes. Extracellular staining consisting in CD45, HLA-DR, CD14, CD64, CD11c, CD103, CD172 (SIRPα) was then incubated for 20 minutes at 4°C. Finally, cells were washed in RPMI medium before being acquired in a FACS ARIA sorter (BD Biosciences).

Sorter gating strategy includes discarding doublets -comparting FSC-A and FSC-H-. Within singlets, total viable leukocytes were identified as $CD45^+$ and negative for the viability dye. Total MΦ are identified as $CD14^+CD64^+$. Within the non-MΦ fraction, total cDC are identified as $HLA\text{-}DR^+CD11c^+$ and can be further divided into subsets based on the expression of CD103 and SIRPα. Type 1 cDC are defined as $CD103^+SIRP\alpha^-$ while type 2 cDC are $SIRP\alpha^+$. Finally, type 2 cDC can be further divided into $CD103^-SIRP\alpha^+$ and $CD103^+SIRP\alpha^+$ (Figure 4.1). Separated cells were automatically collected with the sorter device into 1.5 mL Eppendorf tubes (TermoFisher Scientific, cat # 11926955) containing 1 mL of AIM-V™ medium.

3.3.4 Unsupervised analysis

The OMIQ Data Science platform (©Omiq, Inc. 2022) was utilized after transforming the data, with the scale, parameters, and cofactors configured as recommended by the platform. The PeacoQC algorithm was applied for data cleaning to remove outlier events in spectral cytometry files caused by abnormal flow behaviour, such as clogs or other technical issues. Following this, a manual filtering step was performed to exclude cell debris, doublets, and non-viable cells, retaining only viable myeloid antigen presenting cells (mAPC) ($CD45^+$ $HLA\text{-}DR^+$ cells) for further analysis.

For exploratory analysis, an unsupervised approach using the uniform manifold approximation and projection (UMAP) algorithm was used. This algorithm employs a non-linear, graph-based method to represent multidimensional data and reconstructs it into a two-dimensional map while preserving the multidimensional structure. By doing so, it identifies similarities between cells across all dimensions, which correspond to the intensity of marker expression. The resulting two-dimensional map reflects the proximity of cells

based on their distances in multidimensional space, ensuring that cells with similar expression patterns are positioned close to each other. Prior to analysis, a subsampling step was performed to ensure equal representation across groups.

Subsequently, the FlowSOM algorithm was applied to identify and group similar cell clusters in an unsupervised manner. This algorithm evaluates the expression of all selected markers in each cell and organizes them into metaclusters based on their expression levels. While it enables the visualization of typical biological groupings, it also facilitates the detection of novel or unexpected clusters. However, FlowSOM primarily highlights metaclusters representing major immune system subsets present in the sample. The visual representation of the two algorithms (UMAP and FlowSOM) allows for further subdivision of these metaclusters into smaller clusters, providing a more detailed representation of the phenotypic and functional diversity within mAPCs.

A clustered heatmap was generated using the clusters identified in the previous step. This heatmap visually depicts the expression levels of each phenotypic marker within each cluster, with dendrograms grouping clusters and markers based on similarity. This approach enables the identification of mAPC subsets associated with each cluster by analysing their marker expression profiles. If a specific cluster is linked to a particular condition under study, its phenotype can be further characterized using classical supervised methods, potentially revealing subsets that might otherwise remain undetected. Finally, the refined results from the FlowSOM algorithm were overlaid onto the UMAP plot to visualize their spatial distribution.

3.4 CELL CULTURE

3.4.1 Lamina propria mononuclear cells culture

Total LPMC were cultured in 96-well round bottomed plaques (ThermoFisher, cat # 10418623) in complete AIM-V™ medium (Gibco, cat # 12055091) at a concentration of 200.000 cells in a volume of 200µl. LPMC were cultured 18h with 5% CO_2 at 37ºC. In some cases, LPMC were cultures without stimuli (resting conditions), but in others, cells were cultures with different stimulus: 100 ng/ml lipopolysaccharide (LPS) (Invitrogen, cat # 00-49-76-93), 100 nM tofacitinib citrate (Sigma-Aldrich, cat # PZ0017) or a combination of two (LPS 100 ng/ml and 100 nM tofacitinib citrate). Following 18-hour culture, LPMC were harvested for flow cytometry staining.

3.4.1.1 T cell co-cultures

Different subsets of previously sorted intestinal cDC were used to stimulate naïve T cells in a 1:20 proportion in round bottomed 96-well plaques (ThermoFisher, cat # 10418623) for 5 days in AIM-V™ medium (Gibco, cat # 12055091) and in a final volume of 200 µl. In all cases, experiments included a negative (naïve T cells cultured in resting conditions) and a positive control (T cells stimulated in the presence of 1µg/ml phytohemagglutinin (TermoFisher, cat # 10576015). Naïve T cells were also cultured with paired intestinal Mϕ from the same donor as a second negative controls. Following culture, T cells were harvested for flow cytometry staining.

3.5 INTESTINAL MICROENVIRONMENT EVALUATION

5.1.1 Biopsies-culture supernatants

Cell-free culture supernatant from the biopsy cultures were collected and stored at −80°C until analysed. Prior to analysis, samples were centrifuged to remove any debris. Levels of IFN-γ, IL-10, IL-17A, IL-1β, IL-6, TNF-α, and IL-8 were determined using MILLIPLEX® MAP custom magnetic bead panel kit following the manufacture's specifications. Briefly, the protocol involved preparing assay buffer, calibrators, controls, and magnetic beads coated with specific capture antibodies. After pre-wetting the plate, samples, standards, and controls were incubated with the bead mixture to allow antigen binding. Following incubation and washing steps, detection antibodies and streptavidin-phycoerythrin (SA-PE) were added to generate fluorescence signals. A broad sensitivity range of standards were used to help enable the quantitation of a wide dynamic range of cytokine concentrations while still providing high sensitivity. The plate is read on a Luminex® platform, which detects and quantifies fluorescence for each analyte. Median fluorescent intensity was calculated using the MAGPIX® system version (Luminex) which integrates the Luminex xPOTENT® acquisition software and the MILLIPLEX® Analyst 5.1 analysis software.

3.6 MICROBIOTA ANALYSIS

3.6.1 DNA extraction

3.6.1.1 Fecal DNA Extraction and sequencing

Bacterial DNA was extracted following manufacturer's recommendations for the QIAmp PowerFecal Pro DNA Kit (Qiagen, cat # 51804). Briefly, the protocol involves the efficient extraction of microbial DNA from fecal samples using mechanical and chemical lysis. The process begins with bead-beating to disrupt microbial cells, followed by chemical lysis to enhance DNA release. Then, inhibitors are removed through specialized binding and wash steps, ensuring high-purity DNA. The lysate is then passed through a silica-based spin column, where DNA binds selectively while contaminants are washed away. Finally, the DNA is eluted in a low-salt buffer, yielding high-quality DNA suitable for downstream applications. DNA samples were quantified using a NanoDrop microvolume spectrophotometer (Thermo Fisher, USA) and then kept at -20°C until shipment.

Metagenomic sequencing was performed at the Agricultural Technology Institute of Castilla y León (ITACYL) in Myseq platform (Illumina, San Diego, CA, USA) and using the Nextera XT Index Kit paired-end (2x300nt) (Illumina, San Diego, CA, USA) for microbiota analysis by amplifying the hypervariable V3 and V4 regions of 16S rDNA with specific primers (515F-806R).

3.6.1.2 Biopsy DNA Extraction and sequencing

Human intestinal biopsies were used to analysed both bacteria and fungi. DNA extraction and sequencing was carried out on Seqplexing (Sequencing Multiplex, Valencia) using an Illumina MiSeq device, paired-end 2x250bp. For bacteria (16S DNA), the primers used for amplification were 16S V1-V2 Forward Primer 5'-1: TNANACATGCAAGTCGRRSG; 16S V1-V2 Forward Primer 5'-2: TAACACATGCAAGTCRACTYGA and 16S V1-V2 Reverse Primer 3': GCTGCCTCCCGTAGGAGT. For fungi (ITS2 region), the primers used were ITS2_F1: GTGARTCATCGAATCTTTG, ITS2_R1: TCCTCCGCTTATTGATATGC and ITS2_R2: GATATGCTTAAGTTCAGCGGGT. Biopsies were used also to study viral composition using a Shotgun approach also in Seqplexing (Sequencing Multiplex, Valencia).

3.6.2 Bioinformatic analysis

Data analysis was firstly performed using Qiime2 (https://qiime2.org/), an open-source software, to obtain the necessary datasets to perform the further analysis using R (https://www.r-project.org/). All the code related to this project is available in the following GitHub repository: https://github.com/mariagpms/Microbiome-Analysis.git

Qiime2 workflow started importing raw sequences into Qiime2 using the *q2-tools import* script with the input format *PairedEndFastqManifestPhred33V2*. A file containing the necessary data was previously generated. Then, samples were demultiplexed using the *demux summarize* command, which classifies total reads based on flanking oligonucleotides. After that, DADA2 algorithm was used to remove sequencing errors and distinguish true biological sequences from artifacts. Sequence quality was then assessed, ensuring good read integrity, and low-quality regions, barcodes, and primers were removed using the *dada2 denoise-paired* script. Subsequently, representative sequences were aligned using MAFFT via *q2-alignment*, and taxonomy was assigned to the sequences using a Naïve Bayes classifier (*feature-classifier fit-classifier-naive-bayes* command) against the SILVA 16S v138_99 database for bacteria and UNITE database for fungi, with a 99% similarity threshold in both cases. Relative abundances of all taxes at the genus level were also obtained with the *feature-table relative-frequency* command. Finally, beta diversity analyses were performed by calculating the distance matrix with different distances (Bray-Curtis, Jaccard, UniFrac and Weighted Unifrac) using the command *beta-group-significance* and generating different Principal Coordenate Analysis (PCoA) plots for each beta-diversity distance.

In the R studio analysis, first, microorganisms were associated with their relative abundances in the dataset. Then, the mean frequency for every genus (except the NA's) was calculated in each dataset. Top 15 most abundant genus of each dataset were identified and the frequencies of the 15 most abundant genus were relativized between 0 and 100 to represent them using the *ggplot2* package (library) in a stacked bar plot. This was done for fungi and bacteria found in biopsies as well as bacteria found in stool samples. Moreover, the alpha diversity was measured using the function *estimate_richness* from the *phyloseq* library, using Shannon's and Simpson's indices, to assess species richness and evenness. These values were then plotted into an alpha diversity plot with *ggplot2* library, one for each of the following groups: bacteria in biopsies, bacteria in stools and fungi in biopsies. In addition, the Kruskal Wallis test was performed using Shannon's and Simpson's indices to check whether the median of the different study groups was the same.

Finally, fungus-bacterium correlations were analyzed using Spearman's correlation coefficient and identifying statistically significant associations with its rank test using the *cor_test* function from the library *rstatix*. Then, a heatmap for each study group was plotted with *ggplot2* library.

3.7 STATISTICAL ANALYSIS

Statistical analyses were conducted using GraphPad Prism version 8 for Windows (GraphPad Software, www.graphpad.com) and R (https://www.r-project.org/). For comparisons between two groups, a T-test was performed on normally distributed data, while the Mann-Whitney U test was used for non-parametric data. One/Two-way ANOVA (with or without repeated measures) was applied to compare multiple groups with normal distributions, while the Kruskal-Wallis test was used for non-parametric groups. Pearson correlation was used for normally distributed data, and Spearman correlation for non-parametric data. A p-value of <0.05 was considered statistically significant.

Chapter 4. Study and isolation of human intestinal dendritic cell and macrophage subsets

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4.1 INTRODUCTION

Dendritic cells (DC), the most potent antigen-presenting cells (APC), determine the outcome (pro-inflammatory or tolerogenic) of antigen-specific adaptive immune response (1). DC are divided into two major subsets: conventional or classical DC (cDC) and plasmacytoid DC (pDC). In the gastrointestinal (GI)-tract, cDC are essential to maintain the balance between tolerance towards nutrients/commensals and immunity against pathogens (2). cDC can be further divided into subsets based on their ontogeny and function named type 1 (cDC1), specialized in cross-presentation and type 2 (cDC2), specialized in classical presentation (3). In mice, cDC1 express surface marker CD8α, although in human they are characterized by the expression of CD141. Nevertheless, both mice and human cDC1 express XCR1 and CD103. cDC1 require IRF4 and Notch2 transcription factors, cDC2, on the contrary, require IRF8 and Baft3. While murine cDC2 express surface marker CD11b, in human they are characterized by the expression of CD1c while both species express SIRPα. The gut, however, harbors a unique subset of $CD103^+$ cDC2. This gut-specific population controls most of the mechanisms of immune tolerance given its unique capacity to generate gut-homing regulatory T cells (Treg) and IgA-producing B cells, although they can also drive Th17 responses (4,5).

As opposed to cDC, which can initiate adaptive specific immune responses by performing antigen presentation to naïve T cells, macrophages (MΦ) are APC highly adapted to the tissue that they inhabit (6). In the intestine, MΦ are highly specialized to avoid overt immunity in response to the gut microbiota (7). In human, GI-MΦ can be divided into two major subsets, newly arrived pro-inflammatory monocytes and tissue resident tolerogenic MΦ, based on the expression of CD11c, CCR2 or CXCR3 respectively (8,9).

In this chapter, we will provide therefore the required protocols and guidelines to study human intestinal dendritic cell and macrophage subsets by flow cytometry (either in fresh or following culture) using biopsy explants obtained during routine endoscopy. Moreover, we shall also explain how to enrich them from tissue resections to obtain enough numbers of these scarce cell types to perform further downstream applications including cell culture or “omic” analyses (e.g. RNAseq, proteomics, etc.) among others.

4.2 STUDY OF DENDRITIC CELLS AND MACROPHAGES FROM INTESTINAL BIOPSIES

1 Intestinal biopsies are collected during routine endoscopy in a tube with ice-chilled Roswell Park Memorial Institute (RPMI) Medium. A minimum of 4 biopsies should be obtained to properly characterize human intestinal cDC and MΦ.

2 Biopsy explants should be immediately transferred to the research laboratory within the following 30 minutes to be further processed. Tissue will be incubated with 5 ml Hank's buffered salt solution (HBSS) supplemented with 1mM Dithiothreitol (DTT) and 1mM Ethylenediamine-tetra acetic acid (EDTA) in an orbital shaker (30 minutes, 250 rpm, 37°C). Following incubation, supernatant will be discarded, and the remaining tissue will be incubated under the same conditions to remove the mucus layer, enterocytes and intraepithelial leukocytes.

3 Remaining tissue will be subsequently digested in 5 ml of RPMI medium supplemented with collagenase D (1 mg/mL), liberase (20 µg/mL) and benzonase (25 U/mL) in an orbital shaker (three incubations, 30 minutes each, 250 rpm, 37ºC). Following each incubation, the medium must be filtered with a 100 µm strainer to obtain lamina propria mononuclear cells (LPMC) which will be preserved at 4ºC until used. The remaining tissue will be further digested two more times following the same approach.

4 Following the incubations, LPMC are collected in the same tube which will be further centrifuged (300g, 10 min, 4ºC) and resuspended in RPMI medium.

5 LPMC cells can be directly stained to characterize freshly obtained cDC and MΦ subsets in different patient cohorts by flow cytometry. Alternatively, LPMC can be further cultured (e.g. in resting conditions and in the presence of different compounds) to assess how cDC and MΦ subsets respond to the stimuli.

5.a. Staining for cytometry approach:

5.a.i. Cells are washed to remove RPMI by centrifugation at 300g, 5min, 4ºC. LPMC pellet will be washed in 1 ml of Fluorescence Activated Cells Sorting Buffer (FACS buffer), which consists in Phosphate Buffered Saline (PBS) supplemented with 2% fetal bovine serum (FBS), 3mM NaN_3 and 1 mM EDTA, and further centrifuged (300g, 10 min, 4ºC).

5.a.ii. Pellet will be resuspended in 1ml of FACS with the presence of a viability dye to exclude dead cells from the analysis. Time and titration of the viability dye must be determined within each laboratory to optimize the timing and dilutions. LPMCs will be further washed.

5.a.iii. Pellet will be resuspended with 100µl of FACS buffer in the presence of 2µl of a non-specific Fc Receptor-mediated fluorescent antibody blocker

(FcBlock). LPMC will be incubated 10 min at room temperature (RT) in the dark and then further washed.

5.a.iv. Pellet will be resuspended in FACS buffer and the extracellular antibodies added at the adequate titration (which depends on the clone, the brand and the batch). To study human intestinal cDC and MΦ subsets, in addition to the viability dye, LPMC must be stained with CD45, CD14, CD64, CD103, CD172 (SIRPα), HLA-DR and CD11c (Figure 4.1). In addition, monocytes and MΦ and be further discriminated based on the expression of CD11c (Figure 4.1) (8,9). Moreover, the phenotype of the different cDC and MΦ subsets can be further studied by adding further antibodies to the cocktail to assess their activation status (CD80, CD86, etc.), migration capacity (CCR2, CCR9, etc.). LPMC will be incubated for 20 minutes at 4ºc and then, further washed in FACS buffer

5.a.v. In addition, intracellular staining of the cells at this stage (not discussed) can be also performed to assess the expression of different intracellular markers like transcription factors, cytokine production, etc.

5.a.vi. Following incubation, LPMC are resuspended in 250 µl of 2% paraformaldehyde solution (in PBS) to fix the cells, followed by a further incubation at 10 min at 4ºC. Cells will be subsequently washed with FACS buffer.

5.a.vii. Finally, LPMC will be resuspended in 500 µl of FACS buffer and preserved at 4ºC until acquisition in a cytometer within 48 hours.

5.a.viii. Once acquired in the cytometer, gating strategy includes discarding doublets -comparting FSC-A and FSC-H (not shown)-. Within singlets, total viable leukocytes are identified as $CD45^+$ and negative for the viability dye (Figure 4.1). Total MΦ are identified as $CD14^+CD64^+$ and can be further divided into newly arrived pro-inflammatory monocytes or tissue resident macrophages based on the expression of CD11c, CX3CR1 or CCR2 respectively (8) (Figure 4.1). Within the non-MΦ fraction, total cDC are identified as $HLA\text{-}DR^+CD11c^+$ and can be further divided into subsets based on the expression of CD103 and SIRPα. Type 1 cDC are defined as $CD103^+SIRP\alpha^-$ while type 2 cDC are $SIRP\alpha^+$. Finally, type 2 cDC can be further divided into $CD103^-SIRP\alpha^+$ and $CD103^+SIRP\alpha^+$, being the latter specific to the gastrointestinal tract.

5.b. LPMC culture:

5.b.i. LPMC are washed in RPMI medium and resuspended in AIM-V® serum-free medium.

5.b.ii. LPMC are further cultured in flat-bottom plates at 1.000.000 cells/ml density.

5.b.iii. Different wells can be now supplemented with different conditions including specific antigens (i.e. 33-mer peptide, gliadin…), pro-inflammatory cytokines as IL-15 or TNFα, pro-inflammatory compounds (i.e. LPS), anti-inflammatory compounds, etc. Optimum dose for each stimulus should be optimized based on the specific readout. It is also important to highlight that all experiments must include a negative control or basal condition (i.e. cells cultured in the absence of any stimuli).

5.b.iv. LPMC are incubated overnight (O/N) at 37ºC, 5% CO_2.

5.b.v. Next day, LPMC are recovered from the plates and stained as explained in point a).

4.3 DENDRITIC CELL AND MACROPHAGE ISOLATION FROM INTESTINAL RESECTIONS

1 Human resections are collected immediately following surgery in ice-chilled RPMI medium (4ºC). Typically, around 10g of tissue is enough to perform subsequent processing and enrichment.

2 Once in the laboratory, resections are further prepared to process them. First step is cleaning the tissue with HBSS and remove the muscle and fat using surgical scissors. Resulting tissue is composed of two layers: mucosa (brown, dotted appearance) and submucosa (pink, very irrigated). As long as tissue is clean, cut it into pieces of about 1 cm^2 in order to process them separately as previously described (10).

3 When LPMC are isolated, they can be stained to identify different cDC and MΦ subsets. Hence, total LPMC are stained as above, and the same gating strategy (Figure 4.1) is set on the sorter to enrich total MΦ as well as the three different cDC subsets.

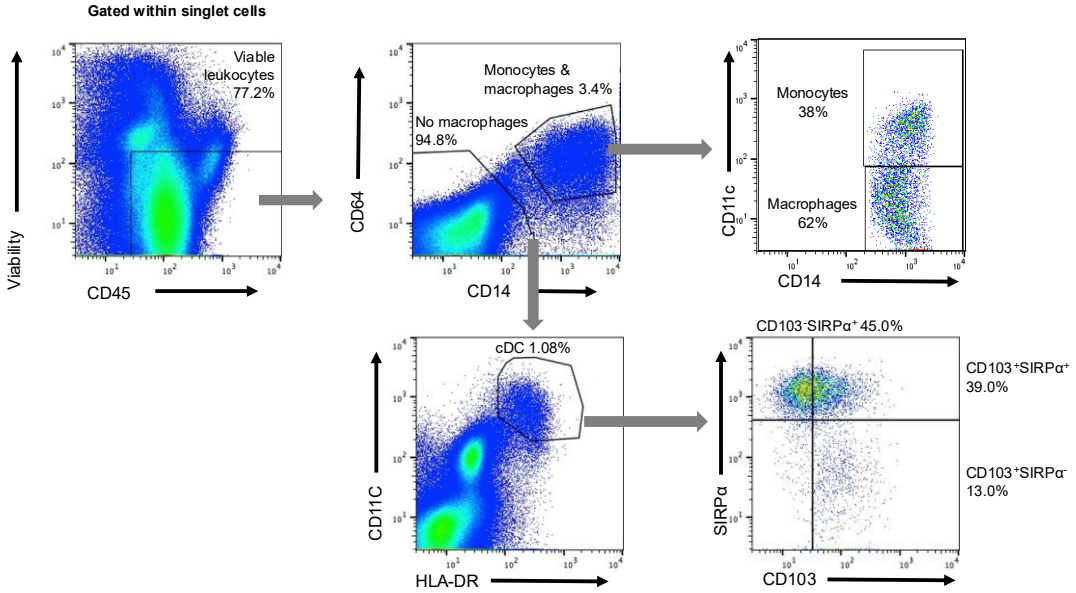

Figure 4.1. Gating strategy to identify conventional dendritic cells and macrophages in the human gut.
Following obtention of lamina propria mononuclear cells from biopsy explants or tissue resections, total leukocytes are identified within the singlet factor as $CD45^+$ which are negative for the viability marker. Within then, total macrophages can be identified as $CD14^+CD64^+$ being all of them $HLA\text{-}DR^+$. These cells can be further divided into newly arrived pro-inflammatory monocytes and tissue resident macrophages based on the expression of CD11c, CCR2 or CX3CR1 respectively (9). Within the non-macrophage gate, total conventional dendritic cells (cDC) are identified as $HLA\text{-}DR^+CD11c^+$ and further divided into subsets based on the expression of CD103 and SIRPα.

4 Following sorting enrichment, post-sort analyses should be performed to confirm the purity of the different isolated cell subsets (Figure 4.2).

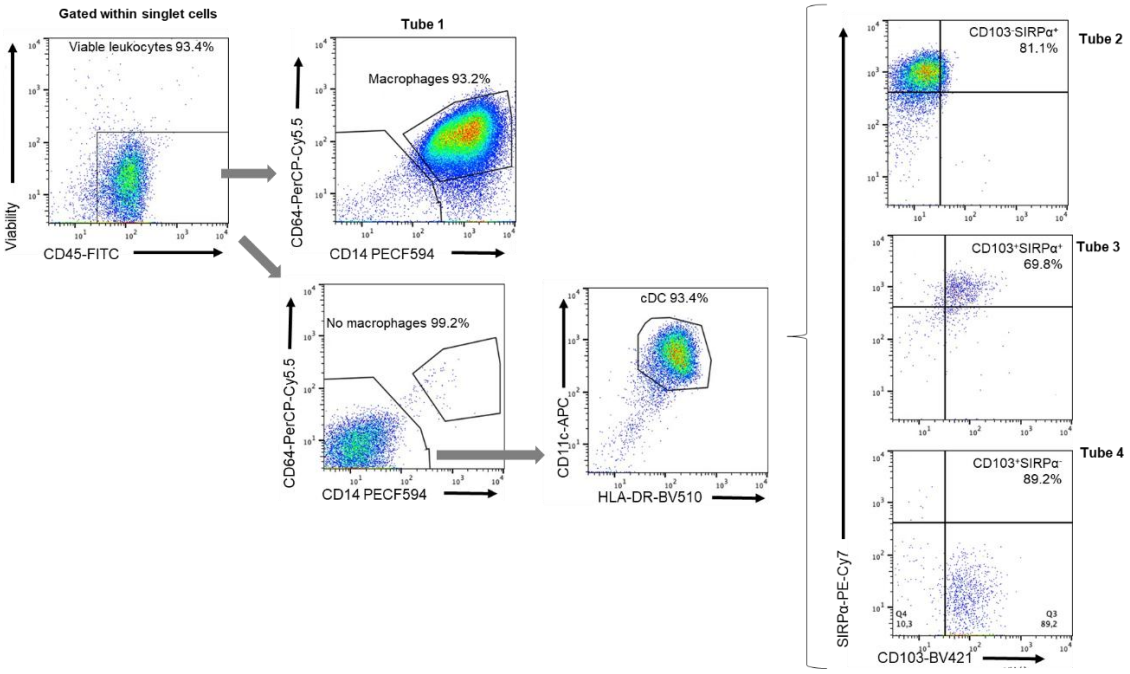

Figure 4.2. Post-sort analysis of human intestinal enriched conventional dendritic cells and macrophages.
Conventional dendritic cell (cDC) subsets and total macrophages are identified as in Figure 4.1 and sorted into 4 different tubes containing total macrophages (Tube 1), CD103$^-$ type 2 cDC (tube 2), CD103$^+$ type 2 cDC (tube 3) and type 1 cDC (Tube 4). Post-sort analysis confirms the purity of the cells within each tube which can be now used for downstream applications.

5 Sorted cells can be now used for further downstream applications including T cell stimulation, pure cell culture with different compounds, proteomics, RNA-seq analyses, etc.

4.4 NOTES

- ✓ It is important to note that for the resections, there is obviously no proper healthy control. In these cases, patients with colorectal cancer can act as controls as far as obtained tissue is at least 10 cm away from the tumor.
- ✓ When LPMC isolation is performed, it is recommended to vortex tubes before and after adding collagenase solution.
- ✓ Three incubations are sufficient to obtain a high number of LPMC from tissue resection even if the tissue is not fully digested although, if needed, further incubations can be performed following the same principles.
- ✓ At the time of performing the sorting enrichment, 1 ml of RMPI should be added to collection tubes before starting the process.
- ✓ Preparing a “stock mix” of antibodies (in the correct dilution) reduces human error and makes experiments more comparable.

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Chapter 5. Type 1 and CD103^{+} type 2 conventional dendritic cells are decreased in active patients with ulcerative colitis but not with Crohn's disease.

Manuscript sent for publication

5.1 INTRODUCTION

Inflammatory bowel disease (IBD), including Crohn´s disease (CD) and ulcerative colitis (UC), is a chronic disorder of unknown aetiology that involves a pathological response of the immune system resulting in chronic inflammation of the gastrointestinal (GI)-tract. IBD prevalence is high, affecting more than 1.6 million inhabitants in the United States and more than 2.2 million in Europe (1). Although IBD incidence varies widely depending on the different countries, it is increasing rapidly, probably due to the "westernization" of lifestyles (2).

Dendritic cells (DC), the most potent antigen-presenting cells (APC), determine the outcome (pro-inflammatory or tolerogenic) of antigen-specific adaptive immune responses (3,4). DC are divided into two major subsets, named conventional or classic DC (cDC) and plasmacytoid DC (pDC). In the GI-tract, cDC are essential to maintain the balance between tolerance towards nutrients/commensals and immunity against pathogens (5–7). cDC can be further divided into subsets based on their ontogeny and function named type 1 (cDC1), specialized in cross-presentation and type 2 (cDC2), specialized in classical presentation (8–10). cDC1 require transcription factors IRF4 and Notch2. In mice, they express surface marker CD8α, although in human they are characterized by the expression of CD141. Nevertheless, both mice and human cDC1 express XCR1 and CD103. cDC2, on the contrary, require transcription factor IRF8 and Baft3. While murine cDC2 express surface marker CD11b, in human they are characterized by the expression of CD1c although both species express SIRPα. The gut, however, harbours a unique subset of $CD103^+$ cDC2. This gut-specific population controls most of the mechanisms of immune tolerance given its unique capacity to generate gut-homing regulatory T cells (Treg) and IgA-producing B cells, although they can also drive Th17 responses (11–13).

While human GI-cDC show a tolerogenic function which helps to maintain immune homeostasis, in IBD patients they display a pro-inflammatory biased profile (14–24). Indeed, GI-cDC from IBD patients have a reduced capacity to generate suppressor Treg cells coupled with an enhanced production of pro-inflammatory Th1 and Th17 effector T cells, which is thought to be associated with lower numbers of $CD103^+$ cDC (15,23,25) . Nevertheless, and although cDC are likely to be essential in IBD development and progression, it is currently unknown whether these differences in cDC biology in IBD are restricted to any particular subset. Moreover, the properties of the immune system (26,27), including cDC subset composition (28–30), systematically change through its length. However, and despite UC exclusively affects the human colon while CD can happen anywhere in the GI-tract (from mouth to anus), few studies attempted to study GI-cDC in both CD and UC abrogating those regional differences. To that end, we here specifically

studied human intestinal cDC from patients with CD or UC, either active or quiescent, and compared them with the non-inflamed colon from the same patients and with controls.

5.2 MATERIAL AND METHODS

5.2.1 Patients and biological samples

Blood (10 ml) and intestinal biopsies from healthy controls were obtained during endoscopy or colonoscopy from a total of 61 healthy controls [37.7% males; 47.7 ± 11.7 years (mean ± standard deviation); age interval 25-80]. Patients had been referred due to rectal bleeding, dyspepsia or colorectal cancer screening. In all cases they had macroscopically and histologically normal mucosa. In the case of a colonoscopy, paired samples were obtained from the distal colon, proximal colon and the terminal ileum (when accessible) from the same patients. Duodenal samples were obtained in the context of an upper endoscopy. A maximum of 8 biopsies were obtained per tissue/patient. Samples were immediately preserved in ice-chilled complete medium [Dutch modified RPMI 1640 (Sigma-Aldrich, Dorset, UK) containing 100 µg/mL penicillin/streptomycin, 2 mM L-glutamine, 50 µg/mL gentamicin (Sigma-Aldrich) and 10% foetal calf serum (TCS Cellworks, Buckingham, UK)] and processed within 30 minutes.

Blood and colonic biopsies were also obtained from IBD patients, including 15 patients with active UC (defined by a Mayo endoscopic score >1; Table 5.1), 11 patients with quiescent UC (defined by a Mayo endoscopic score ≤1;Table 5.2), 12 patients with active CD (defined by a simplified endoscopic activity score for CD (SES-CD) score > 3; Table 5.3) and 7 patients with quiescent CD (defined by a SES-CD score ≤3;Table 5.4). In the case of patients with active disease (either UC or CD), both the inflamed and the non-inflamed colonic mucosa were sampled.

In addition, ileal and colonic resection were obtained from the proximal and distal ends from patients with colorectal cancer, with a minimum distance of 10cm to the tumours (Table 5.5). In a similar manner, the affected and non-affected tissue was also obtained from patients with CD subjected to tissue resection (Table 5.6).

Finally, blood samples from healthy controls were also obtained from the blood bank of Valladolid.

In all cases, samples were obtained following written informed consent after ethical approval from the Ethics Committee at La Princesa Hospital (Madrid, Spain) and Hospital Clínico Universitario (Valladolid, Spain).

Table 5.1. Patient demographics with active ulcerative colitis including gender, age, Mayo Endoscopic score and treatment.

Code	Gender	Age	Mayo Endoscopic	Treatment
1	Male	48	3	Infliximab
2	Female	43	2	Mesalazine + Azathioprine
3	Female	42	2	Prednisolone + Etrolizumab
4	Male	30	3	Infliximab + Adalimumab
5	Female	59	3	Mesalazine
6	Female	29	3	Mesalazine + Azathioprine
7	Female	38	2	Mesalazine
8	Female	31	3	Mesalazine
9	Male	59	2	Mesalazine
10	Female	60	2	Adalimumab
11	Female	60	2	Adalimumab
12	Female	39	3	Adalimumab + Azathioprine
13	Male	42	3	Azathioprine + Mesalazine + Infliximab
14	Female	27	3	Mesalazine + Azathioprine + Golimumab
15	Male	29	3	Untreated

Table 5.2. Patient demographics with quiescent ulcerative colitis including gender, age and treatment.

Code	Gender	Age		Treatment
1	Female	60		Mesalazine
2	Female	58		Mesalazine
3	Female	50		Mesalazine
4	Female	56		Mesalazine
5	Female	23		Metronidazol
6	Female	58		Mesalazine
7	Female	54		Mesalazine
8	Male	59		Mesalazine
9	Male	44		Infliximab
10	Female	55		Mesalazine
11	Female	54		Mesalazine + Azathioprine

Table 5.3. Patient demographics with active Crohn´s disease including gender, age, simplified endoscopic activity score for Crohn´s disease (SES-CD) and treatment.

Code	Gender	Age	SES-CD	Treatment
1	Female	53	9	Untreated
2	Female	36	8	Azathioprine + Vedolizumab
3	Male	59	14	Azathioprine
4	Female	56	5	Vedolizumab
5	Female	62	15	Mesalazine
6	Female	55	5	Untreated
7	Female	65	6	Untreated
8	Male	31	12	Azathioprine + corticoids
9	Female	36	6	Azathioprine + Adalimumab
10	Male	43	9	Sulfasalazine
11	Female	81	9	Adalimumab
12	Female	48	3	Methotrexate

Table 5.4. Patient demographics with quiescent Crohn´s disease including gender, age and treatment.

Code	Gender	Age	Treatment
1	Female	51	Sulfasalazine + Mercaptopurine
2	Male	46	Azathioprine
3	Female	64	Metotrexate + Adalimumab
4	Female	26	Sulfasalazine + Ustekinumab
5	Male	57	Azathioprine + Adalimumab
6	Female	49	Sulfasalazine + Mercaptopurine
7	Female	35	Azathioprine + Infliximab

Table 5.5. Patient demographics with colorectal cancer subjected to intestinal resection and used as controls, including gender and age.

Code	Gender	Age	Type	Behaviour
1	Female	87	Cancer	Non-affected
2	Female	44	Cancer	Non-affected
3	Male	85	Cancer	Non-affected
4	Female	71	Cancer	Non-affected
6	Male	80	Cancer	Non-affected
11	Male	65	Cancer	Non-affected
12	Male	85	Cancer	Non-affected

Table 5.6. Patient demographics with active Crohn's disease subjected to intestinal resection including gender, age and type.

Code	Gender	Age	Type	Behaviour
5	Female	47	Crohn	B3 (penetrating)
7	Female	57	Crohn	B2, B3 (stricturing and penetrating)
8	Male	72	Crohn	B2 (stricturing)
9	Female	41	Crohn	B1 (inflammatory)
10	Male	46	Crohn	B3 (penetrating)
13	Male	53	Crohn	B2 (structuring)

5.2.2 Blood processing

Peripheral blood mononuclear cells (PBMC) were obtained by centrifugation over Ficoll-Paque PLUS (Amersham Biosciences, Chalfont St. Giles, UK). PBMC were washed twice in PBS containing 1 mM Ethylenediaminetetraacetic acid (EDTA) and 0.02% sodium azide (FACS buffer) and stained with fluorochrome-conjugated antibodies as explained below.

5.2.3 Biopsy processing

Intestinal biopsies were processed as previously described (30,31). Briefly, intestinal biopsies were processed to obtain lamina propria mononuclear cells (LPMC) following two incubations (30 minutes each) with Hanks balanced salt solution (HBSS) (Gibco BRL, Paisley, Scotland, UK) containing 1 mM DTT and 1 mM EDTA solutions to remove the associated mucus/bacteria and epithelial layer, respectively, and further digested in the presence of 1 mg/mL of collagenase D and 20 µg/mL of liberase (Roche Diagnostics Ltd, Lewes, UK). LPMC were subsequently passed through a 100 µm cell strainer and collected by centrifugation before they were further used for flow cytometry staining or culture (5 million LPMC in 2.5 mL of complete medium during 18 hours) in the presence/absence of LPS (100 ng/ml, Sigma-Aldrich).

5.2.4 Tissue resections processing

Human intestinal resections were collected immediately following surgery in ice-chilled RPMI medium (4ºC). Tissue was cleaned with HBSS and muscle and fat were removed using surgical scissors. As long as tissue was clean, it was cut it into pieces of about 1 cm^2 in order to process them separately in 15 ml tubes. First, tissue was incubated with 5 ml Hank's buffered salt solution HBSS supplemented with 1mM DTT and 1mM EDTA in an orbital shaker (30 minutes, 250 rpm, 37°C). Following incubation, supernatant was discarded, and remaining tissue was incubated under the same conditions to remove the

mucus layer, enterocytes and intraepithelial leukocytes. The remaining tissue was subsequently digested in 5 ml of RPMI medium supplemented with collagenase D (1 mg/mL), liberase (20 µg/mL) and benzonase (25 U/mL) in an orbital shaker (three incubations, 30 minutes each, 250 rpm, 37ºC). Following each incubation, medium must be filtered with a 100 µm strainer to obtain LPMC which were further preserved at 4ºC until used. Remaining tissue was further digested two more times following the same approach. Following incubations, all LPMC were collected in the same tube which was further centrifuged (300g, 10 min, 4ºC) and resuspended in RPMI before they were cryopreserved in freezing media (FBS supplemented with 10% DMSO) until used.

5.2.5 Human colonic cytokine milieu.

Cell-free culture supernatant from the biopsy cultures were collected and stored at −80°C until analysed. Prior to analysis, samples were centrifuged to remove any debris. Levels of IFN-γ, IL-10, IL-17A, IL-1β, IL-6, TNF-α, and IL-8 were determined using MILLIPLEX® MAP custom magnetic bead panel kit following the manufacture's specifications. A broad sensitivity range of standards were used to help enable the quantitation of a wide dynamic range of cytokine concentrations while still providing high sensitivity. Median fluorescent intensity was calculated using the MAGPIX® system version (Luminex) which integrates the Luminex xPOTENT® acquisition software and the MILLIPLEX® Analyst 5.1 analysis software. Values above or under the standard curve for each cytokine (IFN-γ: 0.61-2,500 pg/mL; IL-10: 0.88-6,000 pg/mL; IL-17A: 0.73-3,000 pg/mL; IL-1β: 0.49-2,000 pg/mL; IL-6: 0.8-2,500 pg/mL; IL-8: 0.31-2,000 pg/mL; TNF-α: 0.43-1,750 pg/mL) were reported as equal to them respectively.

5.2.6 Antibody labelling

Cells were stained with monoclonal antibodies and characterized by flow cytometry. In all cases, a Live/Dead fixable near-IR dead cell stain kit (Molecular Probes) was added to the cells prior to perform antibody staining hence allowing the exclusion of dead cells from the analysis. Table 5.7 shows the specificity, clone, fluorochrome and source of the antibodies used. Cells were labelled in FACS buffer on ice and in the dark for 20 min following Fc block incubation (Becton Dickinson). For the assessment of intracellular cytokines, cells were permeabilized (Leucoperm, Abd Secrotec) following surface staining and stained with intracellular antibodies. cDC endocytic activity was determined by uptake of TRITC-dextran (molecular weight 40 kDa, 100 µg/ml, 30 minutes at 37 °C or on ice; Sigma, UK) followed by subsequent surface staining of the cells as above. In all cases, cells were further washed

in FACS buffer, fixed with 2% paraformaldehyde in FACS buffer for 10 minutes on ice, and washed again in FACS buffer before they were stored at 4 °C prior to acquisition on the flow cytometer. For cell sorting, LPMC were immediately acquired following staining in FACS buffer and collected in complete medium.

Table 5.7. Specificity, clone, conjugate and manufacturer of the different monoclonal antibodies used in the present work.

Antibody Specificity	Clone	Conjugate	Manufacturer
CCR7	5F4	APC	Biolegend
CD3	HIT3a	PE-Cy5	BioLegend
CD4	SK3	BV510	Becton Dickinson
CD11c	BU15	Alexa700	Biolegend
	BU15	APC	invitrogen
CD14	MOP9	PECF594	Becton Dickinson
CD16	3G8	BV786	Becton Dickinson
	3G8	APC-Cy7	Becton Dickinson
CD19	HIB19	PE-Cy5	Becton Dickinson
CD40	5C3	BV711	Becton Dickinson
CD45	HI30	FITC	BioLegend
CD45RA	HI100	PE-Cy7	eBioscience
CD62L	DREG-56	BV510	BioLegend
CD64	10.1.1	PE-Cy5	Miltenyi
	10.1	PerCP-Cy5.5	BioLegend
CD86	2331 (FUN-1)	BV711	Becton Dickinson
CD103	Ber-ACT8	BV421	Biolegend
CD137L	5F4	APC	Biolegend
CD163	GHI/61	Biotin	Biolegend
CD172a (SIRPα)	REA144	PE-Vio770	Miltenyi
CD172 (SIRPα)	SE5A5	PE-Cy7	BioLegend
CD206	DCN228	APC	Miltenyi
HLA-DR	L243	BV570	Biolegend
	1243	BV510	BioLegend
ICOSL	2D3	PE	Biolegend
IFNγ	4S.B3	APC	BioLegend
IL-1β	JK1B-1	APC	Biolegend
IL-10	JES3-12G8	Biotin	Biolegend
	JES3-9D7	PEDazzle 594	BioLegend
IL-17a	BL168	APC-Cy7	BioLegend
IL23-p19	727753	PE	R&D
PD-L1	MIH1	BV786	Becton Dickinson
SLAN (M-DC8)	DD-1	APC	Miltenyi
Streptavidin		BV605	Becton Dickinson
TNFα	MAb11	BV786	R&D

5.2.7 T cell enrichment and stimulation

Naïve T cells T were enriched from total PBMCs from a buffy coat using magnetic beads following the manufacturer instructions (Pan T Cell Isolation Kit human, Miltenyi Biotec) and further stained with CellTrace™ Violet following manufacturer instructions in AIM-V™ medium. In order to confirm that the enrichment was successfully performed, PBMC and the enriched T naïve cells were stained with an easy panel that included a viability die, CD3, CD45RA and CD62L to identify and measure the percentage of enriched T naïve cells (Figure 5.1). Differed subsets of intestinal sorted cDC subsets were used to stimulate naïve T cells in a 1/20 proportion in round bottomed 96-well plaques for 5 days in AIM-V™ medium. In all cases, experiments included a negative (naïve T cells cultured in resting conditions) and a positive control (T cells stimulated in the presence of 1µg/ml phytohemagglutinin). Naïve T cells were also cultured with paired intestinal Mϕ from the same donor as a second negative controls. Following culture, T cells were further stained as previously explained.

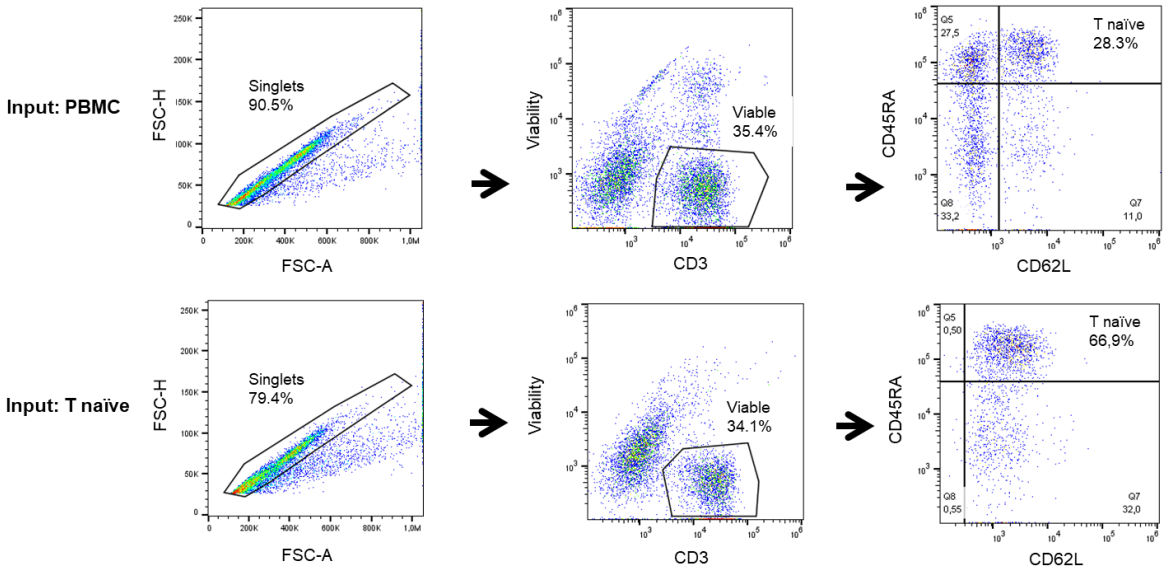


Figure 5.1. T naïve enrichment checking.
T naïve enrichment was checked by staining PBMC and enriched T naïve cells with a simple panel which included a viability die, CD3, CD45RA and CD62L.

5.2.8 Flow cytometry and data analysis

LPMC and PBMC were acquired on a LSR-Fortessa (BD Biosciences) or on a FACS Aria for cell sorting. Following T cell stimulation, cells were acquired on a Gallios (Beckmam Coulter). In cases, results were analyzed using FlowJow (version 10.1). All cells were analysed within singlet viable cells. Positive and negative gatings were set by the fluorescence minus one (FMO) method.

5.2.9 Statistical analysis

T-test or One/Two-way ANOVA (with or without repeated measures) and subsequent Tukey or Sidak ad-hoc correction were applied as detailed in each figure legend. The level of significance was fixed at $p < 0.05$ in all cases.

5.3 RESULTS

5.3.1 CD103$^+$ cDC2 were the main subset in the human duodenum but not in the colon or the ileum.

Human intestinal cDC were identified within singlet viable leukocytes as CD14$^-$CD64$^-$HLA-DR$^+$CD11c$^+$ (Figure 5.2A). Given that the properties of the immune system vary throughout its length (26,27), we first determined cDC densities in different compartments of the human gut revealing that their proportion was higher in the large bowel (proximal and distal colon) compared with the small bowel (terminal ileum and duodenum) (Figure 5.2B).

cDC were divided into subsets based on the expression of CD103 and SIRPα. Hence, cDC1 were identified as CD103$^+$SIRPα$^-$ while cDC2 were identified as SIRPα$^+$. The latter were further divided into CD103$^+$ and CD103$^-$ cDC2 (Figure 5.2C). Further analysis confirmed that cDC1 were CD141$^+$CD1c$^-$XCR1$^+$ while cDC2 were CD141$^-$CD1c$^+$XCR1$^-$ (not shown) in agreement with previous observations (30–32). The proportion of cDC1 and cDC2 did not change throughout the human gut (Figure 5.2D). Further analysis within the cDC2 subset revealed that CD103$^+$ cDC2 were predominant in the duodenum as opposed to the CD103$^-$ cDC2 fraction, which represented the majority of cells in the lower compartments of the GI-tract including the colon and the terminal ileum (Figure 5.2E).

5.3.2 cDC1 and CD103$^+$ cDC2 were more mature than their CD103$^-$ cDC2 counterparts.

We next characterized cDC subsets in the human colon. cDC1 and CD103$^+$ cDC2 were typically more mature than their CD103$^-$ cDC2 counterparts as they had higher levels of CD40, CD137L and ICOSL. HLA-DR and CD86, on the contrary, were preferentially expressed by CD103$^+$ cDC2. Finally, and although CCR7 expression displayed much variability, it was preferentially expressed by cDC1 (Figure 5.3A).

We also assessed PD-L1 expression on human intestinal cDC as it mediates the generation of Treg cells (33,34). Although PD-L1 expression is scarce on human intestinal cDC, its expression was restricted to CD103$^+$ cDC2 (Figure 5.3B). Fc receptor CD16, was also associated with CD103$^+$ cDC2 (Figure 5.3B). However, and although M-DC8/SLAN is associated with CD16$^+$ APC (35), M-DC8/SLAN was not detected on human intestinal cDC or macrophages (MΦ) (data not shown). Scavenger receptors were also differentially expressed between subsets. Hence, although both CD163 and CD206 were found on cDC2, CD163 was preferentially expressed on the CD103$^-$ cDC2 fraction as opposed to CD206 which was mainly found on the CD103$^+$ cDC2 subset (Figure 5.3B). Finally, and

although all cDC subsets were endocytic, that was overall higher on CD103^{+} cDC2 (Figure 5.3C).

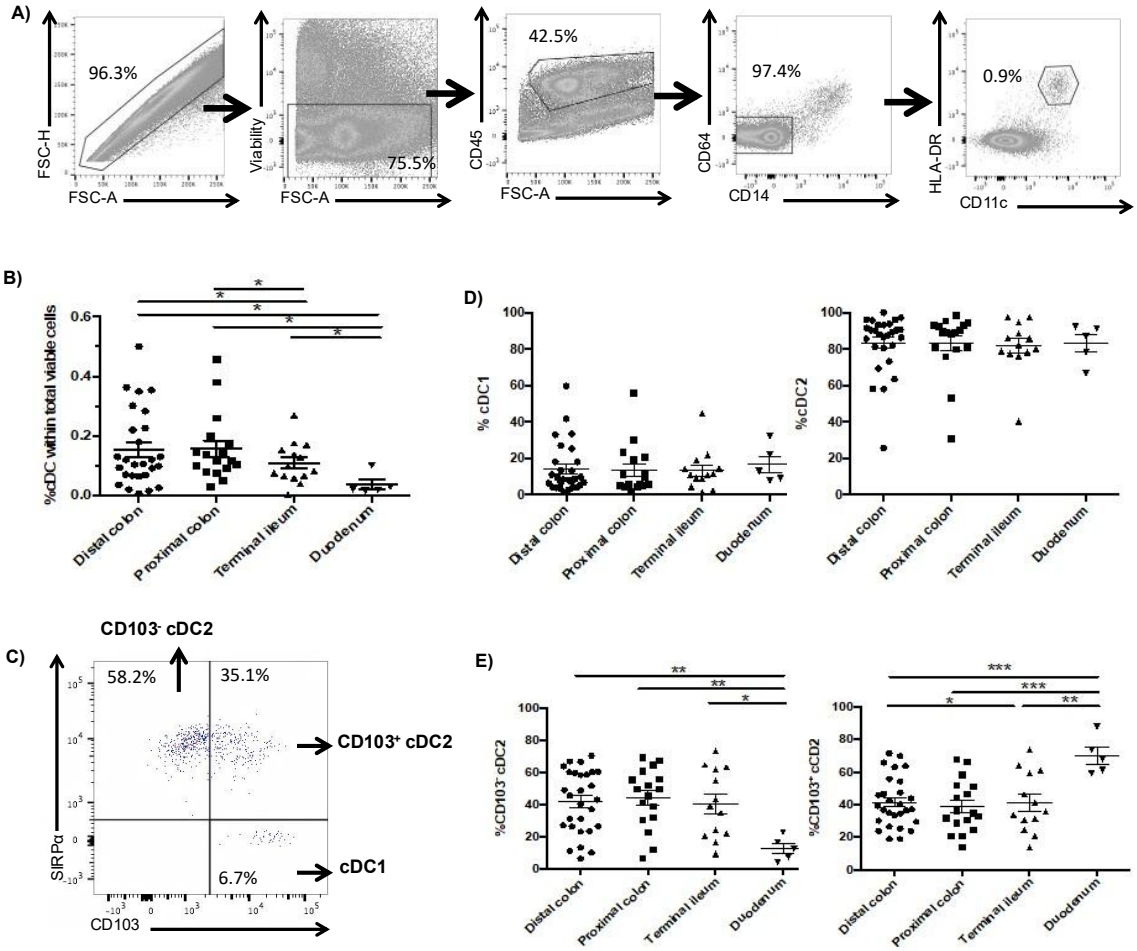


Figure 5.2. Human intestinal conventional dendritic cell subsets.
A) *Human intestinal conventional dendritic cells (cDC) were identified within singlet viable leukocytes (CD45^{+}) as CD14^{-}CD64^{-}HLA^{-}DR^{+}CD11c^{+} on lamina propria mononuclear cells (LPMC) by flow cytometry.* ***B)*** *The proportion of cDC (referred to the total number of viable LPMC) was lower in the duodenum (compared with the ileum, proximal colon and distal colon) and the terminal ileum (compared with both the proximal and distal colon).* ***C)*** *Total cDC were divided into subsets based on the expression of CD103 and SIRPα. Hence, type 1 cDC (cDC1) were identified as CD103^{+}SIRPα^{-} while type 2 (cDC2) were identified as SIRPα^{+}. cDC2 were also divided into subsets based on the expression of CD103.* ***D)*** *cDC1 and cDC2 did not change their proportions though the human gut.* ***E)*** *However, within the cDC2 subset, CD103^{+} cDC2 were the main subset in the duodenum as opposed to the ileum or the colon where CD103^{-} DC2 were the majority. For Figure 5.2B, 1D and 1E samples from the distal colon, proximal colon and terminal ileum were obtained from the same controls (when access to the ileum was available) while duodenal samples were obtained from independent donors. Results from Figure 5.2B, 1D, and 1E also denote samples from the same individuals, considered either total cDC (Figure 5.2B) or divided into subsets (Figure 5.2D and 1E). One-way ANOVA repeated measures and subsequent Tukey´s correction (Figure 5.2B, 1D and 1E) was applied to compare cDC between the distal colon, proximal colon and terminal ileum while duodenal samples were compared with the other three by t-test. P-values <0.05 were considered significant (*p<0.05; **p<0.01; ***p<0.001).*

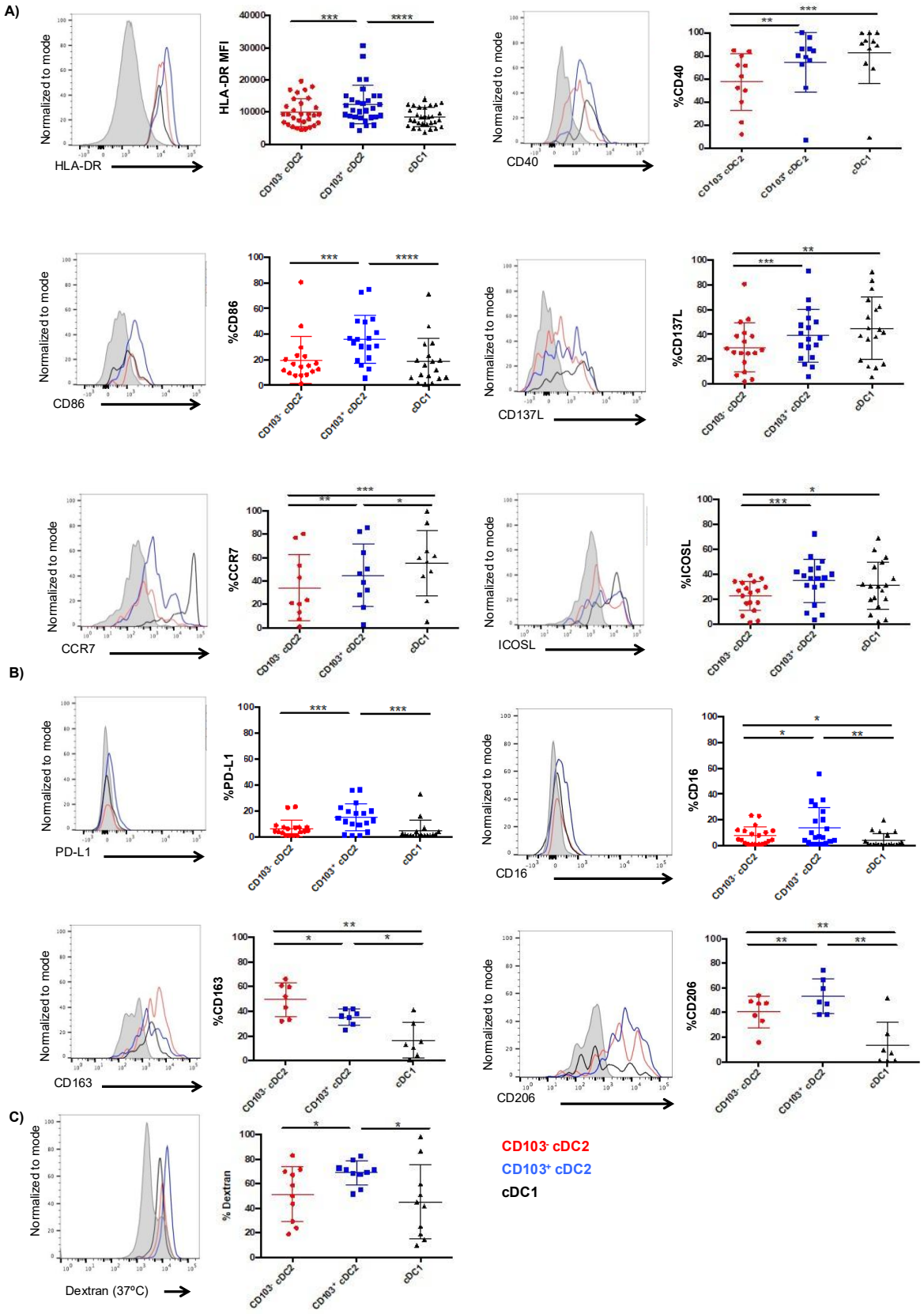


Figure 5.3 Characterization of conventional dendritic cell subsets.
A) *Human intestinal conventional dendritic cell (cDC) subsets were identified as in Figure 5.2C and characterized for the expression HLA-DR, CD40, CD86, CD137L, ICOSL and CCR7; as well as* ***B)*** *PD-L1, CD163, CD206 and CD16.* ***C)*** *DC subset phagocytic capacity was also assessed. Histograms show representative levels of expression of each marker on each given subset. Proportion of positive cells for each given marker within each cDC subset (shown on the pooled plots) was determined by the region method*

*referred to specific fluorescence minus one (FMO) controls (shaded histograms) for all the markers excluding HLA-DR (were shaded histogram denotes its expression on the CD11c$^-$HLA$^-$DR$^-$ fraction within lamina propria viable leukocytes) and Dextran (were shaded histogram shows the phagocytic capacity of cell preserved as 4ºC). One-way ANOVA repeated measures with Tukey´s correction was applied in all cases. P-values <0.05 were considered significant (*p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001).*

5.3.3 The proportion of CD103$^+$ cDC2 was increased following overnight culture.

The ontogeny of CD103$^+$ cDC2 remains unknown, although they are likely derived from newly arrived CD103$^-$ cDC2 following mucosal conditioning (32). In this regard, the proportion of cDC1 and cDC2 DC was not altered following LPMC overnight culture either in resting conditions or in the presence of LPS (Figure 5.4A and Figure 5.4B). However, and within cDC2, the proportion of CD103$^+$ cells was increased after culture, although that was prevented in the presence of pro-inflammatory LPS (Figure 5.4A and Figure 5.4B).

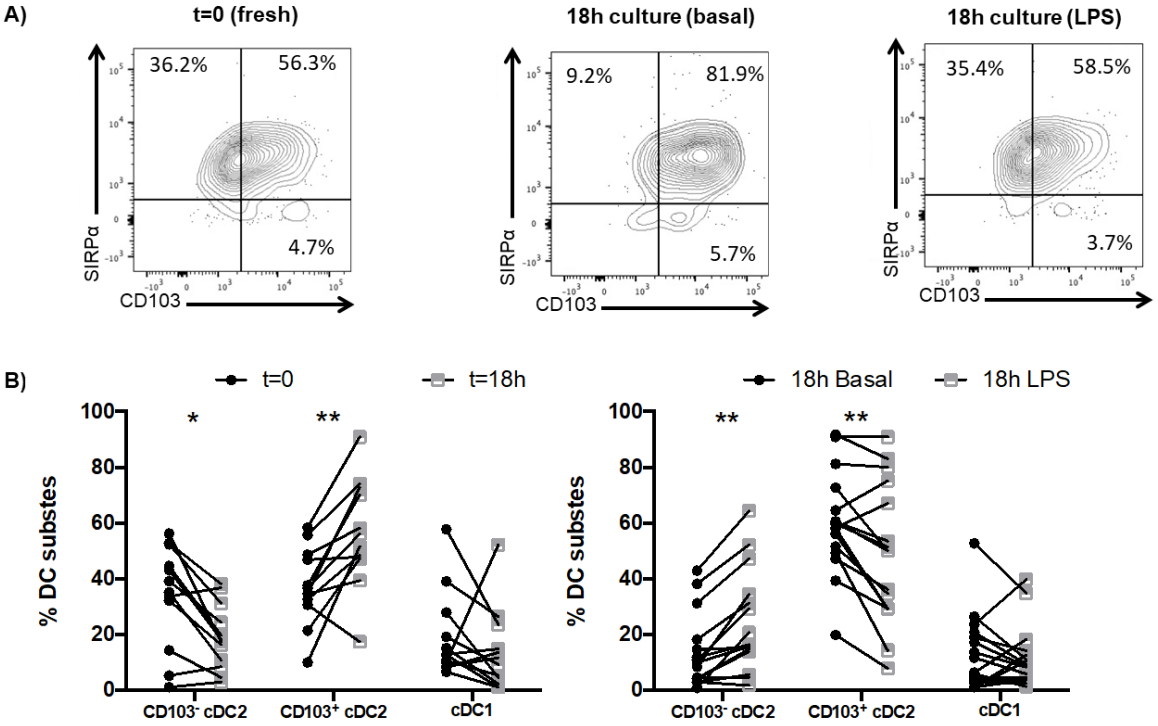


Figure 5.4. CD103$^+$ conventional dendritic cells increase their proportion following culture.
A) *Human intestinal conventional dendritic cells (cDC) were identified as in Figure 5.2C within fresh lamina propria mononuclear cells as well as after overnight culture in resting conditions or in the presence of LPS. Pooled results from several independent experiments are shown in* ***B)****. Two-way ANOVA repeated measures with Sidak correction was applied in Figure 5.4B. P-values <0.05 were considered significant (*p<0.05; ** p<0.01).*

5.3.4 IL-10 production was related to CD103$^+$ DC2.

We next assessed the cytokine profile of the different mucosal cDC subsets. While IL-1β production was associated with cDC2, both in resting condition as well as in the presence of LPS, IL-23 was not produced by any particular cDC subset (Figure 5.5). TNFα

production, on the contrary, was mainly produced by CD103$^+$ cDC2. Finally, IL-10 was produced by all human intestinal cDC subsets, although its production was higher on CD103$^+$ cDC2.

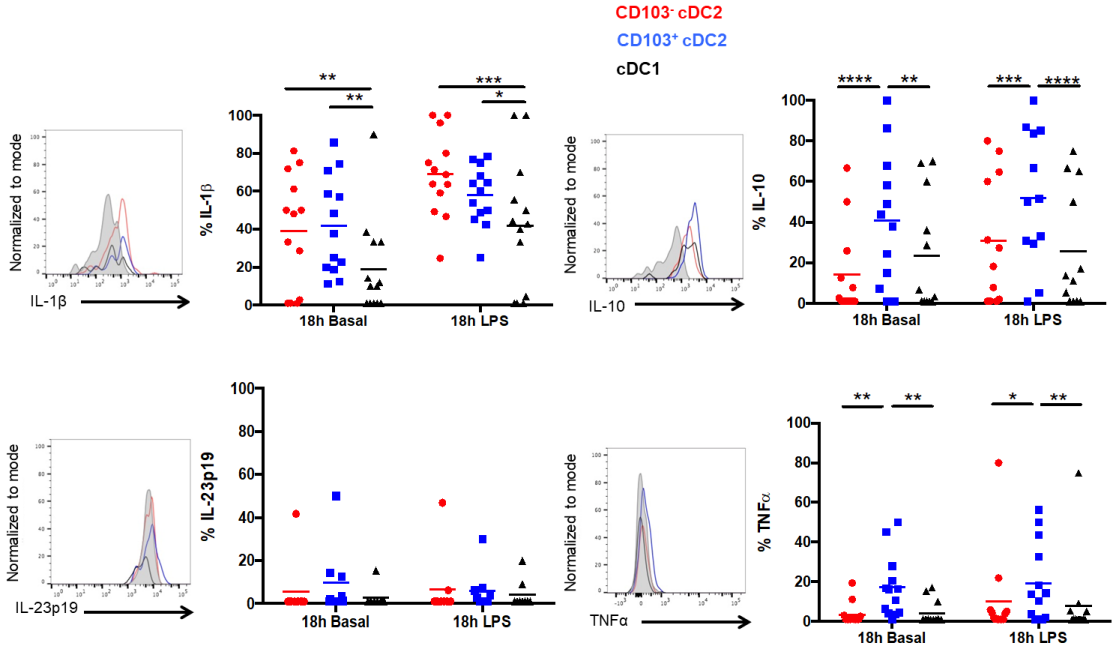


Figure 5.5. IL-10 production is associated with CD103$^+$ type 2 conventional dendritic cells.
*Human intestinal conventional dendritic cell (cDC) subsets were determined as in Figure 5.2C and their intracellular production of IL-1β, IL-10, IL23p19 and TNFα determined. Histograms show representative examples of cytokine production within each given subset. The proportion of positive cells for each given marker within each cDC subset (shown on the pooled plots) was determined by the region method referred to specific fluorescence minus one (FMO) controls (shaded histograms). Experiments were performed after 18 hours culture both in resting conditions (basal) or in the presence of LPS. Two-way ANOVA repeated measures with Sidak correction was applied. P-values <0.05 were considered significant (*p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001).*

5.3.5 All human intestinal cDC subsets prime the generation of IL-10$^+$ T cells in the non-inflamed colon

Having addressed human intestinal cDC phenotype in resting conditions, we next studied their stimulatory capacity in the human setting. To that end, human intestinal CD103$^-$ cDC2, CD103$^+$ cDC2 and cDC1 subsets were sorted in parallel to total Mϕ as previously described (36), and their stimulatory capacity, together with the acquired profile of the stimulated T cells, determined (Figure 5.6). Our results confirmed that all human intestinal cDC subsets can stimulate human allogenic naïve T cells, as opposed to intestinal macrophages (Figure 5.7A). Besides, all human intestinal cDC subsets (including cDC1) primed mainly the proliferation of CD4$^+$ T cells (Figure 5.7B). Indeed, the 3 intestinal cDC subsets primed the generation of IL-10$^+$ T cells, being this capacity expanded in the cDC1 and CD103$^+$ cDC2 with little or no production of IFNγ or IL-17A (Figure 5.7C). In addition and given that the

properties of human intestinal cDC change through its length (28–30), we also determined whether that also translates into a different function between ileal and colonic cDC. Hence, Figure 5.7D proves that all 3 ileal cDC subsets are more stimulatory in the ileum than in the colon, although the type (Figure 5.7E) or cytokine profile (Figure 5.7F) of the responding T cells is not affected by cDC origin.

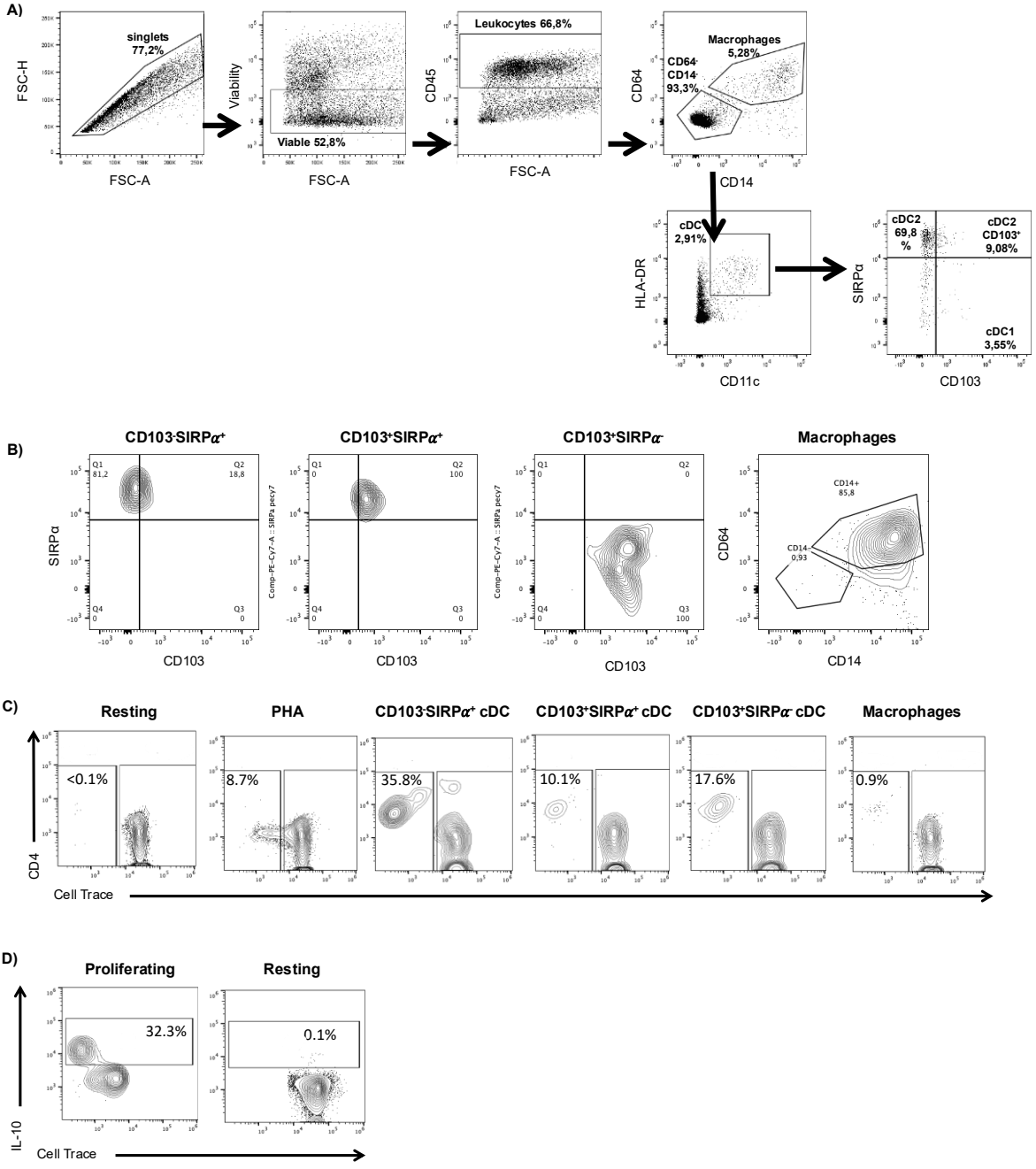


Figure 5.6. Human intestinal dendritic cell stimulatory capacity.
A) *Total human intestinal conventional dendritic cells (cDC) were identified as in Figure 5.2 and divided into subsets based on the CD103 and SIRPα. Total macrophages were also identified, within singlet viable CD45⁺ as CD14⁺CD64⁺. Post-sort acquisition of the 3 different cDC subsets, together with total macrophages is shown in* ***B)****.* ***C)*** *cDC subsets and macrophage stimulatory capacity was assessed on cell-trace labelled allogeneic naïve T cells, as well as the acquired cytokine profile as shown in* ***D)****. Results from one experiment representative of several independent ones is shown.*

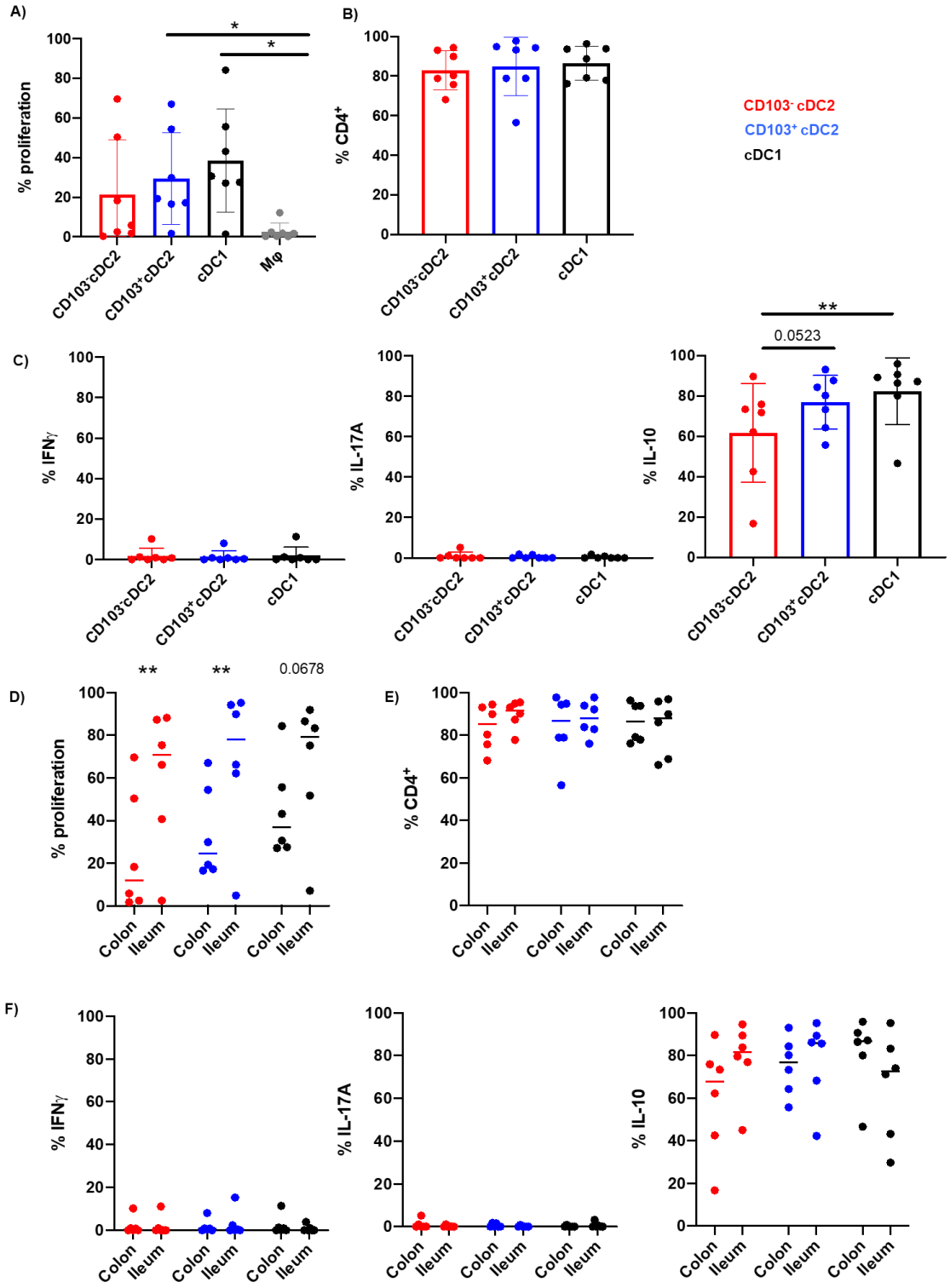


Figure 5.7. All human intestinal conventional dendritic cell subsets prime the generation of IL-10 producing T cells.
A) *Human intestinal conventional dendritic cell (cDC) subsets and macrophages were sorted as in Figure 5.6 and used to stimulate allogenic naïve T cells. The proportion of CD4⁺ T cells that had been stimulated is shown in* ***B)****, while the cytokine profile of the responding CD4⁺ T cells is shown in* ***C). D)*** *Comparison of the proliferation of three cDC subsets both from the colon and the ileum; the percentage of proliferating CD4+ T cells is shown in* ***E)*** *and the cytokine profile of the responding CD4+ T cells is shown in* ***F).*** *Paired One-way ANOVA with Tukey correction was applied in Figure 5.6A-C, while paired Two-way ANOVA with Sidak correction was applied in Figure 5.6D-G. P-values <0.05 were considered significant (*p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001). One-way ANOVA repeated measures with Tukey´s correction was applied in all cases. P-values <0.05 were considered significant (*p<0.05; ** p<0.01).*

5.3.6 SIRPα expression was decreased on mucosal cDC from IBD patients, while the proportion of cDC1 and CD103$^+$ cDC2 was lower in the inflamed colon from UC but not CD patients.

Having characterized the phenotype and function of human intestinal cDC in health, they were further studied in IBD. Given that the proportion of cDC (Figure 5.2B), together with their subset composition (Figure 5.2E) and stimulatory capacity (Figure 5.7E), are influenced by the tissue under study, we here specifically focused on the human colon, hence abrogating regional differences.

The proportion of total colonic cDC (Figure 5.8A) was not altered in the IBD mucosa. Nevertheless, colonic cDC from IBD patients constitutively displayed lower expression of SIRPα irrespectively of the IBD type (UC/CD) or status (inflamed/non-inflamed) (Figure 5.8B and Figure 5.8C). Further analysis shown that the proportion of cDC1 and CD103$^+$ cDC2 were specifically lower in the inflamed colon from patients with active UC but not CD (Figure 5.8D). Consequently, the inflamed tissue from these patients also carried higher numbers of CD103$^-$ cDC2. Moreover, the inflamed mucosa from patients with active UC (and to a lower extent the non-inflamed tissue from the same patients) also displayed higher numbers of CD103$^-$SIRPα$^-$ putative cDC (Figure 5.8D).

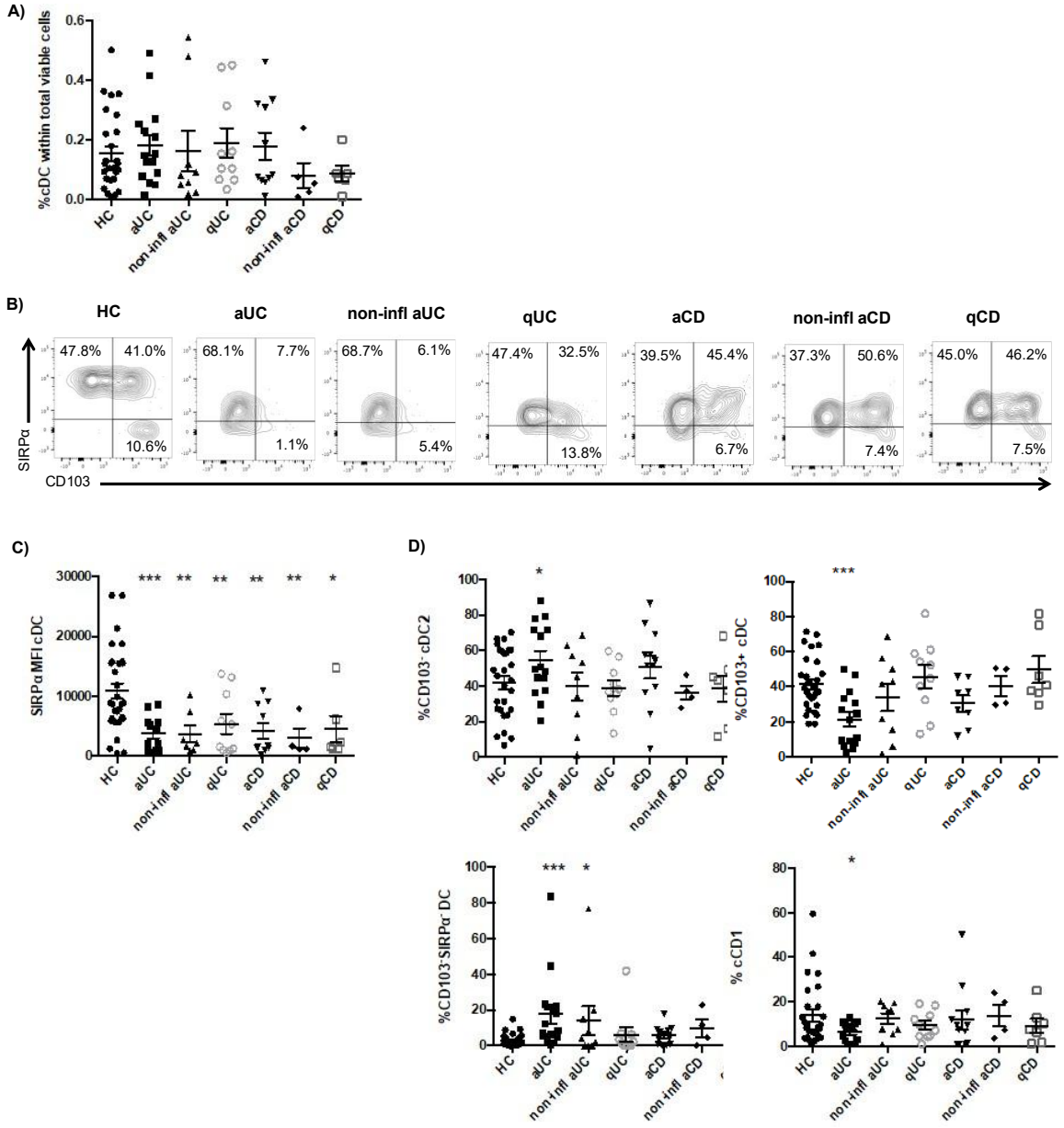


Figure 5.8. Human intestinal conventional dendritic cells display a decreased expression of SIRPα in inflammatory bowel disease coupled with lower numbers of type 1 and CD103⁺ type 2 conventional dendritic cells in ulcerative colitis.

A) *The proportion of human intestinal conventional dendritic cells (cDC), identified as in Figure 5.2, was determined in the colon from healthy controls (HC), in the inflamed tissue from patients with active ulcerative colitis (aUC) as well as in the non-inflamed colon from the same patients (non-infl aUC) and in patients with quiescent ulcerative colitis (qUC). DC proportion was also determined in the inflamed tissue from patients with active Crohn´s disease (aCD) and the non-inflamed tissue from the same patients (non-infl aCD) as well as in patients with quiescent disease (qCD).* ***B)*** *cDC subset composition (based on the expression of CD103 and SIRPα) was also determined in the colonic mucosa from the same patients as in Figure 5.2C. Pooled results regarding the intensity of SIRP on total cDC, as well as the proportion of the different cDC subsets, are shown in* ***C)*** *and* ***D)*** *respectively. One-way ANOVA with Dunnett´s correction was applied in Figure 5.8C and Figure 5.8D. Ad-hoc comparisons were performed, in all cases, compared with healthy mucosa. P-values <0.05 were considered significant (*p<0.05; ** p<0.01; *** p<0.001).*

5.3.7 Dysregulated phenotype on intestinal cDC subsets from IBD patients.

We next assessed the cDC phenotype in the IBD mucosa. HLA-DR expression was decreased on CD103$^+$ cDC2 in the inflamed colon from patients with CD and UC compared with the healthy mucosa (Figure 5.9). Activation markers CD40 and ICOSL were increased on cDC2 from patients with active disease (CD or UC), although CD40 was higher in the CD103$^-$ fraction as opposed to ICOSL which was expanded in the CD103$^+$ subset (Figure 5.9). CD137L was associated with active CD as it was higher in all 3 subsets (CD103$^-$ cDC2, CD103$^+$ cDC2 and cDC1) as well as on CD103$^+$ cDC2 from patients with quiescent CD (Figure 5.9). On the contrary, CD86 was associated with inflamed UC as it was increased on all cDC subsets from these patients as well as on CD103$^-$ cDC2 in CD. Finally, inhibitor receptor PD-L1 was ubiquitously up-regulated on all intestinal cDC subsets in the inflamed colon from IBD patients.

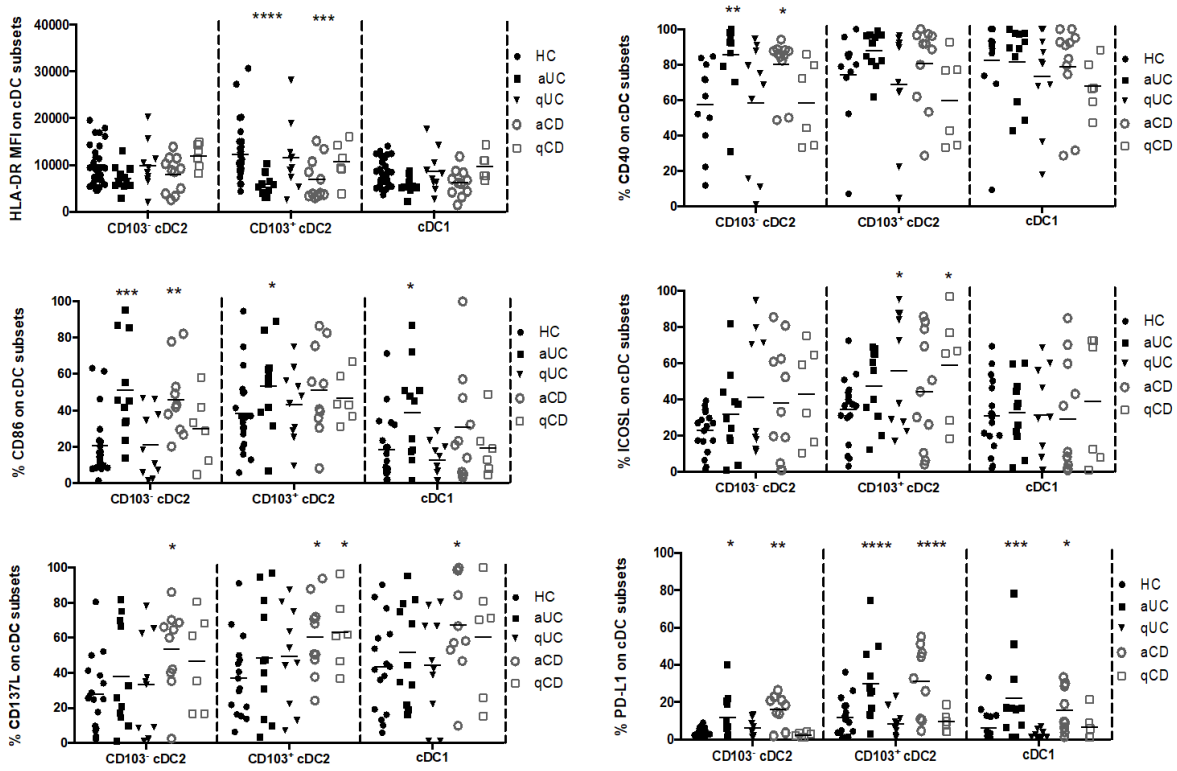


Figure 5.9. Characterization of human conventional intestinal dendritic cell subsets in inflammatory bowel disease.
*Human intestinal conventional dendritic cells (cDC) subsets were identified as in Figure 5.2. The expression of HLA-DR, CD40, CD86, ICOSL, CD137L and PD-L1 on each cDC subset was further determined as in Figure 5.3 in the colonic mucosa from healthy controls (HC) as well as in the inflamed colon from patients with active ulcerative colitis (aUC) or Crohn´s disease (aCD) together with the non-inflamed mucosa from patients with quiescent ulcerative colitis (qUC) or Crohn´s disease (qCD). One-way ANOVA with Tukey correction was applied in Figure 5.9A, while Two-way ANOVA with Sidak correction was applied in Figure 5.9B. Ad-hoc comparisons were performed, in all cases, compared with the healthy mucosa. P-values <0.05 were considered significant (*p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001).*

5.3.8 cDC stimulatory capacity in IBD

Having proved that human intestinal cDC subsets display an altered composition and phenotype in IBD patients (which was however more prominent in UC patients), we next determined whether that also translated into a different function. Given however, the UC patients barely have resections, the stimulatory capacity of human intestinal cDC subsets was therefore restricted to CD patients.

Our results revealed that, in agreement with the altered phenotype elicited from the non-inflamed tissue from CD patients, cDC showed an increase stimulatory capacity as elicited over allogenic naïve T cells (Figure 5.10A) although that did not translate into a differential cytokine profile (Figure 5.10B). Nevertheless, and although cDC subsets from the inflamed ileum from CD patient did not display such increased stimulatory capacity (Figure 5.10C), likely due to the increased stimulatory capacity that ileal cDC already have (Figure 5.7E), CD103$^+$ cDC2 from such tissue had an increased capacity to generate IL-17$^+$ helper T cells (Figure 5.10D).

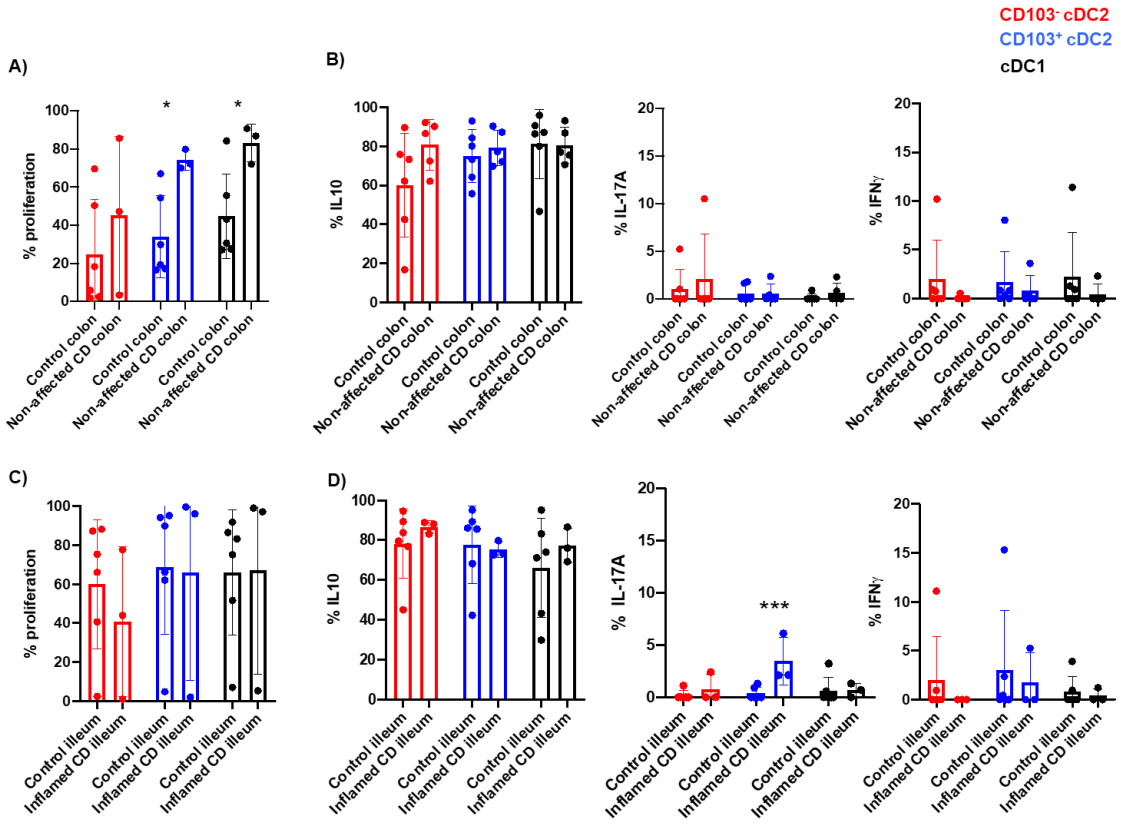


Figure 5.10. Human intestinal conventional dendritic cells are more stimulatory in inflammatory bowel disease and prime the generation of IL-17$^+$ T cells in the inflamed tissue.
A) *Human intestinal conventional dendritic cell (cDC) subset stimulatory capacity from the colon from controls and the non-inflamed colon from patients with Crohn´s disease (CD) was assessed as in Figure 5.6 and Figure 5.7, while the acquire cytokine profile of the CD4$^+$ responding T cells is shown in* ***B)****.* ***C)*** *cDC subsets stimulatory capacity and* ***D)*** *the induced cytokine profile of the CD4$^+$ responding T cells was also determined in the ileum from controls, as well as in the inflamed tissue from CD patients. Two-way ANOVA with Sidak correction was applied. P-values <0.05 were considered significant (*p<0.05; *** p<0.001).*

5.3.9 Cytokine profile in colonic biopsies

The analysis of cytokine concentrations in colonic biopsies revealed significant differences between supernatants from HC and patients with IBD, depending on disease activity and type. In aUC supernatants, there was a pronounced increase in pro-inflammatory cytokines, including IFN-γ, IL-1β, IL-6, IL-8, IL-17, and TNF-α, compared to HC ($p < 0.05$ for all). Additionally, anti-inflammatory IL-10 was significantly elevated in aUC. In supernatants from aCD inflamed mucosa, IL-1β and IL-8 cytokines were significantly increased; INFγ, IL-6 and TNFα were elevated, but the response was less pronounced compared to aUC. In non-inflamed mucosa from quiescent status of IBD (both qUC and qCD), most cytokine levels returned to baseline, except for a slight persistence of IL-6 in qCD (Figure 5.11).

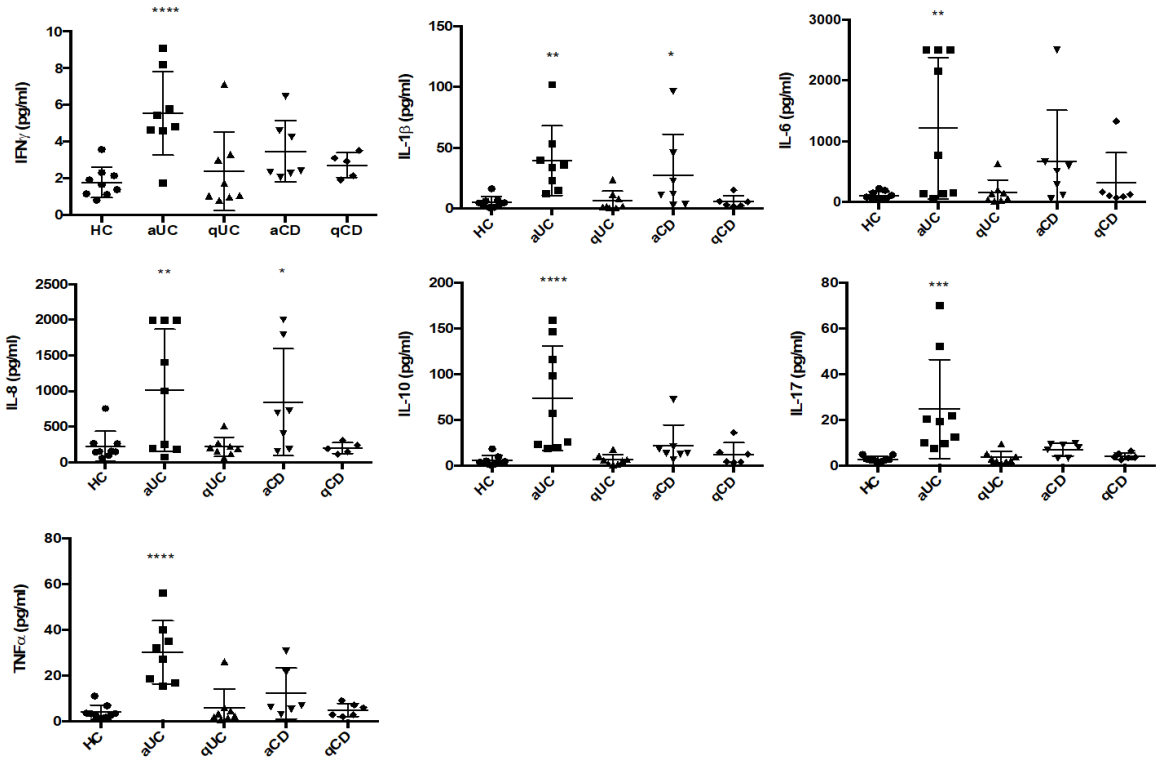


Figure 5.11. Colonic cytokine milieu in inflammatory bowel disease.
*Colonic biopsies from healthy controls (HC), together with the inflamed colon from patients with active ulcerative colitis (aUC) or Crohn´s disease (aCD) and the non-inflamed mucosa from patients with quiescent ulcerative colitis (qUC) or Crohn´s disease (qCD) were cultured overnight in complete medium before multiplex cytokine analysis was performed. Those cytokines with values above or below the standard curve were reported as equal to the limits. One-way ANOVA with Tukey correction was applied. Ad-hoc comparisons were performed, in all cases, compared with the healthy mucosa. P-values <0.05 were considered significant (*p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001).*

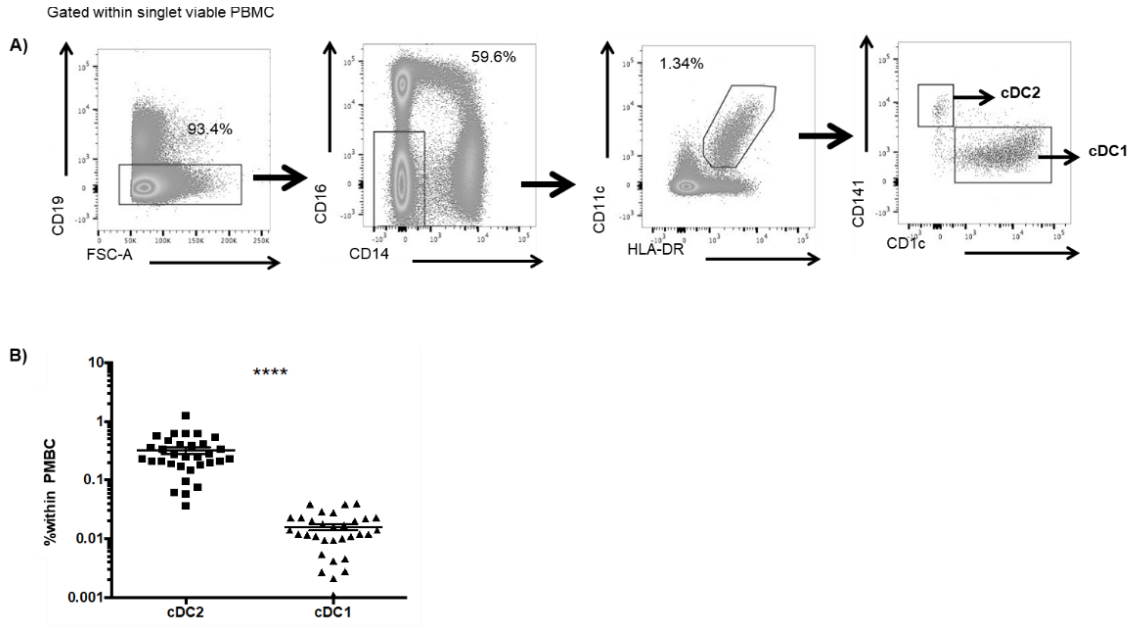
5.3.10 Circulating cDC subsets are not altered in IBD patients.

Conventional blood circulating dendritic cells were identified as $CD14^{-}CD16^{-}CD19^{-}HLA\text{-}DR^{+}CD11c^{+}$ cells from the total PMBC and subsequently divided into two subsets: cDC1

(CD141^{+}) and cDC2 (CD1c^{+}) (Figure 5.12A). Analysis of the relative proportions of these subsets within PBMC revealed that cDC2 was significantly more abundant than cDC1 when pooling data of all groups (Figure 5.12B).

Phenotypic characterization of cDC subsets in HC included the expression of activation and regulatory markers such as HLA-DR, CD40, CD86, CD137L, and PD-L1. cDC2 displayed higher expression of CD86 compared to cDC1, which also showed higher expression of CD40. Levels of HLA-DR, CD137 and PD-L1 were similar between the two subsets (Figure 5.12C).

When comparing the expression of those markers (HLA-DR, CD40, CD86, CD137L, and PD-L1) in the two subsets (cDC1 and cDC2) in IBD groups (aUC, qUC, aCD and qCD) regarding controls, we showed that the proportion of cDC subsets and the expression of most markers (CD40, CD86, CD137L and HLA-DR) remained similar to HC across both cDC subsets among IBD patients. However, PD-L1 expression on cDC2 was significantly increased in patients with aUC compared to HC (Figure 5.12D).



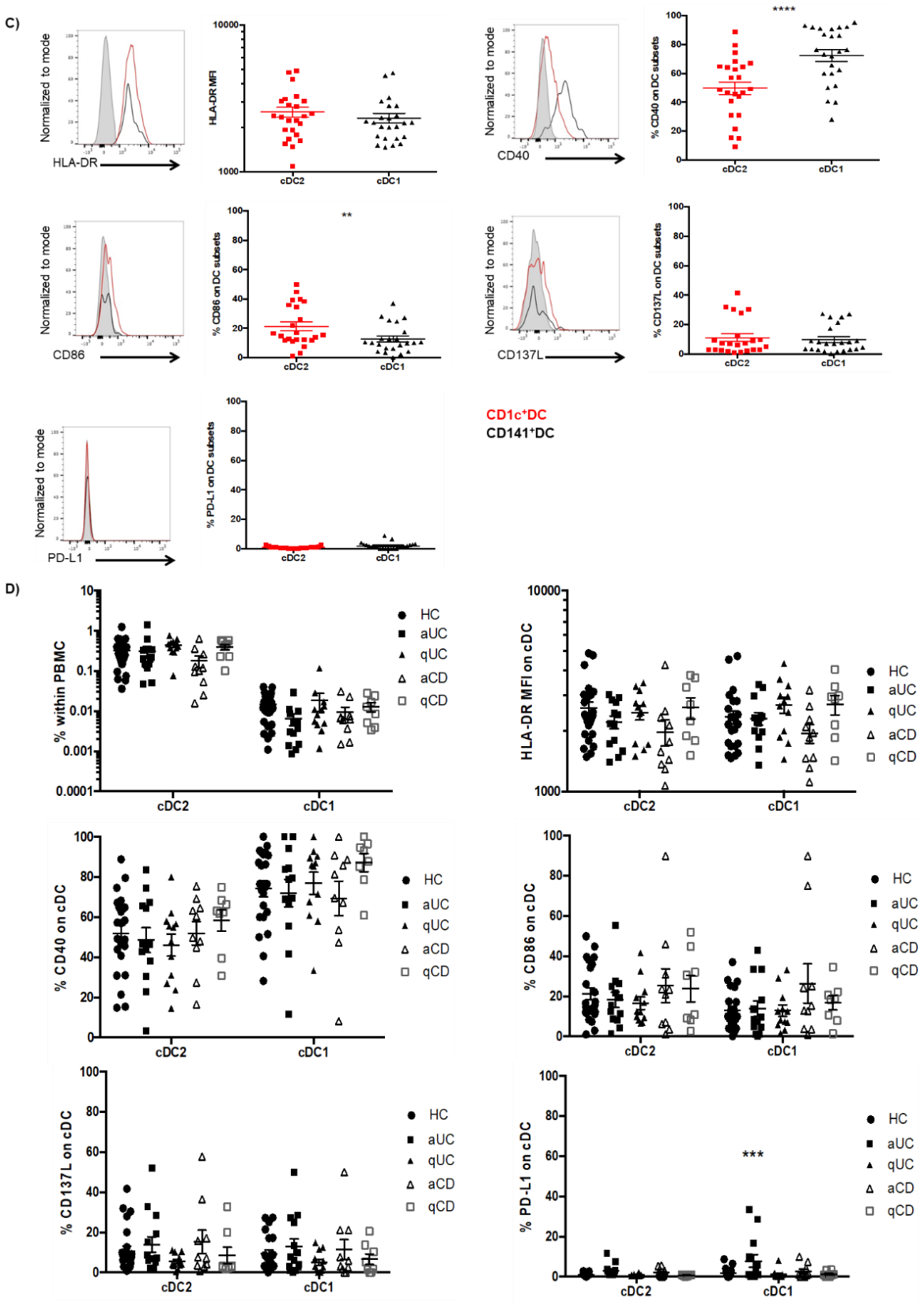


Figure 5.12. Circulating dendritic cells are not altered in IBD patients.
A) *Conventional blood circulating dendritic cells (cDC) were identified within the CD14$^-$CD16$^-$CD19$^-$ fraction as HLA-DR$^+$CD11c$^+$ and further divided into type 1 (cDC1) and type 2 (cDC2) based on the expression of CD141 and CD1c respectively.* ***B)*** *Pooled data shows the relative proportion of both subsets within total peripheral blood mononuclear cells (PBMC).* ***C)*** *Blood circulating cDC1 and cDC2 were characterized in healthy individuals for the expression of HLA-DR, CD40, CD86, CD137L, and PD-L1.* ***D)*** *cDC subsets relative*

*proportion, as well as their phenotype, were also determined in the blood from patients with inflammatory bowel disease, including ulcerative colitis (either active –aUC- or quiescent –qUC-) and Crohn's disease (either active –aCD- or quiescent –qCD-). Histograms show representative levels of expression of each marker on each given subset. Proportion of positive cells for each given marker within each cDC subset (shown on the pooled plots) was determined by the region method referred to specific fluorescence minus one (FMO) controls (shaded histograms) for all the markers excluding HLA-DR (were shaded histogram denotes it expression on the $CD11c^{-}HLA\text{-}DR^{-}$ fraction). Paired t-test was applied on panels B and C. Two-way ANOVA with Sidak correction was applied in panel D. Ad-hoc comparisons were performed, in all cases, compared with the blood DC subsets from healthy controls. P-values <0.05 were considered significant (*p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001).*

5.4 DISCUSSION

Although the mechanisms of immune tolerance in the human gut have been traditionally related to CD103$^+$ cDC2, recent evidence suggests that cDC1 are also needed to achieve such goal (37). Hence, both cCD1 and cDC2 are essential to prime the mechanisms of intestinal tolerance in a retinoic acid dependent manner (11,12). Indeed, the role of PD-L1 on intestinal cDC to maintain the mechanisms of immune homeostasis is more relevant in the small bowel, while in the colon that tolerogenic effect would be XCR1-deppendent (38), in agreement with the restricted PD-L1 expression that we have reported on human intestinal cDC in health. Moreover, we hereby have also showed that all ileal cDC subsets are more stimulatory than their colonic counterparts (28) at the time that have also unveiled how all human intestinal cDC subsets prime the generation of IL-10$^+$ helper T cells, being that capacity increased in CD103$^+$ cDC (both cDC1 and cDC2) in agreement with the regulatory properties attributed to these cells (11,12,28).

Specifically referred to the colon from IBD patients, our results revealed that although the proportion of cDC2 was not altered in these patients, DC from IBD patients constitutively display lower levels of SIRPα irrespectively of IBD type (CD or UC) or mucosal condition (inflamed or non-inflamed). Besides, we have also described how cDC1 and CD103$^+$ cDC2 are specifically reduced in the inflamed colon from UC patients, but not from CD patients, describing therefore a differential immune signature between both conditions. These results confirmed that mucosal cDC from IBD patients display an altered subset composition, phenotype and function, including an increased stimulatory capacity in the non-inflamed colon from IBD patients.

Although the proportion of total cDC changes throughout the human GI-tract (Figure 5.2B), the relative proportion of cDC1 and cDC2 (Figure 5.2D) remains stable through the duodenum, terminal ileum, distal colon and proximal colon. CD103$^+$ cDC2 are predominant in the proximal compartments of the small bowel (duodenum) as previously reported (29). Nevertheless, and although the terminal ileum is technically small bowel, given its proximity to the distal colon is not surprising that it carries a higher proportion of CD103$^-$ cDC2 as in the colon (Figure 5.2E). This is indeed an important consideration for human studies where the terminal ileum (and not the duodenum) is typically used to represent the small intestine. Future studies should therefore be aware that the terminal ileum may be more similar (from an immune point of view) to the colon so it may not represent a good tissue to study the human small bowel as opposed to the duodenum.

It has been previously reported that the numbers of CD103$^+$ cDC are lower in the inflamed colon from patients with IBD, irrespectively of its type (CD or UC) (22) or mucosal status

(23). Here, we have shown how the inflamed colon from patients with UC carried lower levels of both cDC1 and $CD103^+$ cDC2, rendering the inflamed tissue from these patients with a higher proportion of putative $CD103^-SIRP\alpha^-$ cDC. However, the inflamed colon from CD patients with CD did not display a specific reduction of any cDC subset, hence revealing specific differences between CD and UC patients. Besides, and although there was not a specific reduction of $CD103^+$ cDC in CD patients, these cells were more stimulatory, even in the non-inflamed tissue, at the time that they also acquire the capacity to prime the generation of IL-17^+ T cells in the inflamed ileum. Given nevertheless the low proportion of UC patients that require a colectomy, we could not sort enough number of cDC from UC patients in order to address whether they also had a differential function as in CD.

It is currently unknown whether the different cDC subsets present in the mucosa truly represent different subsets or, on the contrary, different developmental or activation stages (10). Recent evidence suggests that, at least in mice, $CD103^+$ cDC2 originate from $CD103^-$ cDC2 after mucosal conditioning in a TGFβ-dependent manner (39). Our results support this idea, as we observed an increase in $CD103^+$ cDC2 after LPMC culture. However, this process is blocked in the presence of pro-inflammatory LPS, likely due to the resulting inflammatory environment, including IL-1β, which inhibits $CD103^+$ DC differentiation in the GI-tract (40). This would also explain the lower proportion of $CD103^+$ cDC2 in the colon of UC patients, but not in CD patients. The more pro-inflammatory cytokine environment in UC (Figure 5.11) may prevent the local differentiation of newly arrived $CD103^-$ cDC2 into $CD103^+$ cDC2.

In agreement with that concept, circulating cDC1 and cDC2 were also studied in the blood from these patients (Figure 5.12A), being the later the main subset in the human blood (Figure 5.12B). Indeed, both subsets displayed differences in their phenotype (Figure 5.12C) although the presence of IBD (either CD or UC, both active or quiescent) did not have any major impact on their phenotype (Figure 5.12D). Hence, these results again suggest that the altered phenotype and function of human intestinal cDC in IBD is acquired once they have entered the tissue, given that the intestinal microenvironment modulates the phenotype and function of intestinal cDC through the action of both hosts, dietary and microbial-derived metabolites (13,41–44). Hence, in the presence of a pro-inflammatory stimuli, like the one found in the IBD mucosa intestinal cDC2, can be reprogrammed to prime the generation of proinflammatory immune responses (45), including the generation of Th17 cells (46,47) as in the inflamed ileum from CD patients.

SIRPα (a regulatory membrane glycoprotein) is predominantly expressed on the surface of APC including cDC and MΦ. Its ligand, CD47, prevents cell phagocytosis by the APC on a

mechanism named as the “don´t eat me signal”. This signalling pathway has been related with cDC regulation and the development of autoimmunity (48) including murine models of IBD where neutralization of the CD47/SIRPα signalling pathway prevents trinitrobenzene sulfonic acid induced colitis (49). In our work, we have described how colonic cDC from IBD patients display lower levels of SIRPα. This is in agreement with previous observations in the context of UC (23), which are hereby expanded to CD. Hence, mucosal cDC from IBD patients carry lower levels of SIRPα, irrespectively of IBD type (CD or UC) or condition (inflamed or non-inflamed). However, blood cDC were classified into cDC1 and cDC2 based on the expression of CD141 and CD1c, respectively (Figure 5.12). Given that SIRPα was not included among the markers used to characterized blood cDC in IBD, we cannot confirm whether such lower expression found in the mucosa is a constitutive difference on cDC biology in IBD, or on the contrary an acquired phenotype once they have entered the tissue. Further studies should identify the specific mechanisms controlling the lower levels of SIRPα shown by mucosal cDC in IBD and determine its functional implications, if any, on IBD pathogenesis.

PD-L1 expression on cDC is required to supress T cells and, in the presence of TGFβ, to generate Treg cells (33,34). Within intestinal cDC, PD-L1 expression was restricted to the $CD103^+$ cDC2 subset, which also display an enhanced capacity to produce IL-10 in agreement with the regulatory functions attributed to this subset in the maintenance of intestinal homeostasis. Nevertheless, in the inflamed IBD mucosa, PD-L1 expression was ubiquitously up-regulated on all intestinal cDC subsets confirming previous observations reporting increased PD-L1 expression within LPMC from CD patients (50) and which, together, may explain the higher numbers of $FOXP3^+$ Treg cells found in the intestinal IBD mucosa (51). Although this process is likely aiming to control the exacerbated immune response found in the inflamed tissue from these patients (52), we cannot discard the possibility that the PD1/PD-L1 signalling pathway is not fully functional in IBD (53).

One of the major limitations of our study is that we are aware of the large individual variability regarding cDC subset composition and phenotype in IBD patients. Nevertheless, we could not find any correlation between these observations and the demographics (gender, age), severity of the inflammation (Mayo endoscopic score for UC and SES-CD for CD) or treatment among the patients suggesting that IBD is a multifactorial disease where unfortunately no simple factors explain the heterogeneity of the disease.

In summary, here we have reported how human intestinal cDC can be divided into different subsets with differences in their phenotype and function, having all of them the capacity to prime the generation of IL-10 T cells. In addition, we have also observed that, in IBD, cDC2

display lower expression levels of SIRPα irrespectively of IBD type (CD or UC) or mucosal condition (inflamed or non-inflamed), while cDC1 and gut specific $CD103^+$ cDC2 are specifically reduced in the inflamed colon from patients with UC but not with CD. This suggests the presence of different pathogenic mechanisms operating between colonic CD and UC, which may translate therefore into the development of better therapies which specifically target the altered routes between CD and UC.

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Chapter 6. Tofacitinib downregulates JAK1 and JAK3 on human intestinal monocytes and macrophages without affecting dendritic cells phenotype or function.

Published manuscript

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6.1 INTRODUCTION

The gastrointestinal (GI)-tract is in contact with a wide variety of commensal microbiota and diverse pathogens. Therefore, it requires a balance between immunity and immune tolerance; the lack of immune responses, or immune tolerance, to food antigens and the commensal microbiota is essential to keep the homeostasis of the GI-tract (1). Inflammatory bowel disease (IBD), which includes ulcerative colitis (UC) and Crohn's Disease (CD), is an inflammatory disorder of the GI-tract characterized by an uncontrolled inflammation and abnormal activation of the immune system that occurs when intestinal homeostasis becomes dysregulated (2). Although the aetiology of IBD remains largely unknown, studies indicate that the individual's genetic susceptibility, external environment, intestinal microbiota and immune responses are all involved and functionally integrated in the pathogenesis of IBD (2,3).

While CD can cause transmural inflammation and affect any part of the GI-tract in a non-continuous manner, UC is typified by mucosal inflammation and limited to the colon (2). Development of targeted gut-specific therapy for IBD is still an unmet need. UC is a serious, costly and persistent health issue with a socioeconomic impact comparable with that for other chronic diseases. Treatment involves escalating drug regimens with concomitant side effects followed by surgical interventions which are often multiple. Indeed, the most effective current available therapies like the biological drugs (antibodies targeting immune mediators like TNFα, α4β7, p40, etc.) are only effective in around 1/3 of patients so there is a need to develop novel and better compounds to treat IBD patients.

The Janus kinase (JAK) family includes four intracellular tyrosine kinases: JAK1, JAK2, JAK3, and one non-receptor tyrosine-protein kinase 2 (TYK2). These proteins associate with the intracellular portion of cytokine or hormone receptors and activate signal transducers and activators of transcription (STATs) through autophosphorylation in an intracellular signal transduction pathway (4). Upon binding of a cytokine or hormone to its receptor, the subunits of receptors form multimers, enabling JAK proteins to phosphorylate the associated cytokine receptor. Phosphorylated intracellular cytokine receptor facilitates recruitment of STATs. JAK proteins phosphorylate STAT proteins, leading to STAT homo-dimerization. The STAT homodimer localizes to the nucleus and activates downstream transcription (4). JAK-STAT pathways regulate signalling for multiple immune-relevant mediators, including type I interferon, IFN-γ, and IL-2, IL-4, IL-6, IL-7, IL-9, IL-12, IL-15, IL-21, IL-23, and IL-27 and they are implicated in the pathogenesis of inflammatory bowel diseases (4,5). JAK signalling pathway plays therefore a critical role in mediating inflammatory immune responses. Specially, JAK3 appears to play an important role in driving lymphocyte development, proliferation, and differentiation as its signalling drives

$CD4^+$ T cell differentiation into specialized Type 1 helper T (Th1) and Type 2 (Th2) (6). Furthermore, IL-15 signalling through JAK3 serves as a survival signal for NK cells (6).

Building from that, Tofacitinib (CP-690550) is an oral, small molecule, Janus kinase inhibitor currently used to treat patients with UC (6–8). Tofacitinib interferes with the JAK-STAT signalling by competing with ATP for binding to the kinase domain of JAKs and inhibits JAK1, JAK2, and JAK3. In vitro studies, however, showed preferential inhibition of JAK1 and JAK3 with less effect on JAK2 (9). Despite knowing this, it remains unknown which type of cells are the specific target of Tofacitinib.

In this regard, antigen presenting cells (APC), including conventional dendritic cells (cDC), monocytes, and macrophages, are essential to maintain the mechanisms of immune tolerance towards nutrients and commensals, and immunity against invading pathogens (1,3). Besides, the JAK-STAT signalling pathway play a key role modulating the phenotype and function of human intestinal APC. (10–13)

Given therefore the central role displayed by the JAK-STAT signalling pathway on cDC, monocytes and macrophages, we hereby aimed to assess the specific contribution of these cells to disease progression in UC and to identify the immunomodulatory effects that Tofacitinib elicits over them.

6.2 MATERIAL AND METHODS

6.2.1 Patients and biological samples

Colonic intestinal biopsies were obtained from patients with UC undergoing a colonoscopy for disease diagnose and/monitoring. A total of 10 patients with active (aUC, defined by a Mayo endoscopic score ≥1; 70% men, 52 ± 16 years) and 10 patients with quiescent disease (qUC, defined by a Mayo endoscopic score =0; 60% women, 59 ± 13 years) were included. Intestinal biopsies from 10 healthy controls (60% women, 77 ± 8 years), referred for colonoscopy due to rectal bleeding, dyspepsia or colorectal cancer screening but with macroscopically and histologically normal mucosa, were also obtained. All samples were obtained at the Digestive Service from both Hospital Clínico Universitario and Hospital Universitario Río Hortega (both from Valladolid, Spain). In all cases, biopsies were preserved in Roswell Park Memorial Institute (RPMI) Medium (Sigma-Aldrich, Dorset, UK) at 4ºC, and processed immediately. Patient demographics including disease condition (active/quiescent), gender, age, Mayo endoscopic score, UCEIS and treatment is shown in Table 6.1.

Ileocolonic resections were also obtained from 10 patients (60% men, 77 ± 8 years) with proximal colon cancer at the General Surgery and Digestive System Service from Hospital Clínico Universitario (Valladolid) following written informed consent from the patients (approval code by the CEIm Area del Salud de Valladolid Este 19-1353). The non-affected tissue (minimum distance of 10cm with the tumour) was preserved in Roswell Park Memorial Institute (RPMI) Medium (Sigma-Aldrich, Dorset, UK) at 4ºC until processed.

Peripheral blood samples were also obtained from healthy controls provided by the “Biobanco del Centro de Hemoterapia y Hemodonación de Castilla y León” (Valladolid, Spain).

Table 6.1. Patient demographics.

Code	Type	Status	Age	Gender	Mayo endoscopic	UCEIS	Treatment
1	Control	Healthy	73	Male	-	-	-
2	Control	Healthy	66	Female	-	-	-
3	Control	Healthy	67	Male	-	-	-
4	Control	Healthy	84	Female	-	-	-
5	Control	Healthy	79	Female	-	-	-
6	Control	Healthy	86	Female	-	-	-
7	Control	Healthy	87	Male	-	-	-
8	Control	Healthy	81	Male	-	-	-
9	Control	Healthy	83	Male	-	-	-
10	Control	Healthy	67	Male	-	-	-
11	UC	aUC	61	Male	1-2	2	Mesalazine
12	UC	aUC	50	Male	3	4-5	Mesalazine
13	UC	aUC	67	Male	1	1-2	Mesalazine
14	UC	aUC	66	Male	1	2	Mesalazine
15	UC	aUC	71	Male	0-1	1	Ustekinumab + Mesalazine
16	UC	aUC	50	Female	2	2	-
17	UC	aUC	27	Male	3	5	Tofacitinib + Mesalazine
18	UC	aUC	29	Male	2	3	Mesalazine + Azathioprine
19	UC	aUC	37	Female	1	1	Infliximab
20	UC	aUC	59	Female	3	4	Mesalazine + Azathioprine
21	UC	qUC	57	Female	0	0	Azathioprine
22	UC	qUC	74	Male	0	0	Mesalazine
23	UC	qUC	39	Male	0	0	Mesalazine
24	UC	qUC	56	Male	0	0	Mesalazine + Azathioprine
25	UC	qUC	75	Male	0	0	Mesalazine
26	UC	qUC	59	Female	0	0	Mesalazine
27	UC	qUC	74	Male	0	0	Mesalazine
28	UC	qUC	50	Female	0	0	Mesalazine
29	UC	qUC	62	Male	0	0	Vedolizumab
30	UC	qUC	42	Female	1	2	Infliximab+ Mesalazine

6.2.2 Sample processing

Once in the laboratory, biopsies were incubated with 5mL Hank's buffered salt solution (HBSS) (Gibco BRL, Paisley, Scotland, UK) supplemented with 1mM Dithiothreitol (DTT) (ThermoFisher Scientific, Waltham, USA) and 1mM Methylenediamine-tetra acetic acid (EDTA) (ThermoFisher Scientific, Waltham, USA) in an orbital shaker (30 min, 250 rpm, 37°C). Following incubation, supernatant was discarded, and the remaining tissue was incubated under the same conditions to remove the mucus layer, enterocytes and intraepithelial leukocytes. Remaining tissue was subsequently digested in 5mL of RPMI medium supplemented with 1mg/mL collagenase D (Roche Diagnostics GmbH, Mannheim, Germany), 20µg/mL liberase (Roche Diagnostics GmbH, Mannheim, Germany) and 25U/mL benzonase (ThermoFisher Scientific, Bonn Germany) in an orbital shaker (three

incubations, 30min each, 250rpm, 37°C). Following each incubation, the medium was filtered with a 100µm strainer to obtain lamina propria mononuclear cells (LPMC) which were preserved at 4°C until used. Remaining tissue was further digested two more times following the same approach. Following incubations, LPMC were collected in the same tube which was further centrifuged (300g, 10min, 4°C) and resuspended in RPMI medium. Human intestinal resections were cleaned with HBSS and muscle and fat were subsequently removed using surgical scissors. Tissue was further cut it into smaller pieces and processed as above.

Peripheral blood samples were processed to obtain peripheral blood mononuclear cells (PBMC) by centrifugation over Ficoll-Paque PLUS (Amersham Biosciences, Chalfont St. Giles, UK).

6.2.3 Lamina propria mononuclear cells culture

Total LPMC from controls were further cultured in complete medium (AIM-V™ medium, Gibco BRL, Paisley, Scotland, UK) in resting conditions, as well as was 100 ng/ml lipopolysaccharide (LPS) (ThermoFisher Scientific, Waltham, USA) in the presence/absence of 100 nM tofacitinib citrate (active principle of Tofacitinib) (Sigma-Aldrich, Dorset, UK) with 5% CO_2 at 37ºC. On the contrary, LPMC from patients with UC (either active or quiescent) were just cultured in the presence/absence of Tofacitinib as above. Following 18-hour culture, cell-free culture supernatants were cryopreserved until further used while LPMC were harvested for flow cytometry staining.

6.2.4 Human intestinal cDC sorting and T cell stimulation.

Following LPMC culture from the tissue resections in resting conditions, as well as in the presence/absence of Tofacitinib following LPS stimulation, total cDC were sorted on a FACS Aria III cell sorter (BD Biosciences, New Jersey, USA) as previously published by our group (14).On the other hand, total T cells from control PBMC were magnetically sorted following the manufacturer instructions (Human Pan T Cell Isolation Kit, Miltenyi Biotec, Bergisch Gladbach, Germany) while subsequent naïve T cell enrichment was performed with the REAlease® CD62L MicroBead (Miltenyi Biotec, Bergisch Gladbach, Germany). Naïve T cells were further stained with the proliferation marker CellTrace™ Violet (ThermoFisher Scientific, Waltham, USA).

Total cDC from each condition were used to stimulate naïve T cells in a proportion of 5%-95% on 96-well plaques for 5 days in AIM-V™ medium. In all cases, T cells were also

cultured in resting conditions as well as with phytohemagglutinin (PHA) 1µg/ml. Following culture, T cells were harvested and stained.

6.2.5 Flow Cytometry staining

Cells were stained using viability dye Near-IR (ThermoFisher Scientific, Waltham, USA) and blocking the unspecific unions with Fc block (BD Biosciences, New Jersey, USA). Table 6.2 shows the specificity, clone, fluorochrome and source of the antibodies used. In all cases, cells were further washed in FACS buffer (PBS (ThermoFisher Scientific, Waltham, USA)) containing 1 mM EDTA and 0.02% sodium azide (Sigma-Aldrich, Dorset, UK)) Intracellular staining was performing after fixation by adding intracellular antibodies with a permeabilizer Fix and Perm™ kit (ThermoFisher Scientific, California, USA). Cells were finally fixed with 1% Buffered Formalin (ThermoFisher Scientific, California, USA) for 10 minutes at 4 °C. Cell were then washed in FACS buffer before they were acquired (within 48 hours).

Table 6.2. Specificity, clone, conjugate and manufacturer of the different monoclonal antibodies used in the present work.

Antigen Specificity	Conjugate	Clone	Manufacturer
CD3	PE-Cy5	HIT3a	BioLegend
CD4	BV510	SK3	Becton Dickinson
CD11c	Alexa Fluor 700	Bu15	BioLegend
	APC	BU15	Invitrogen
CD14	cFluor V450	M5E2	CYTEK
	PECF594	MϕP9	Becton Dickinson
CD45	BUV395	HI30	Becton Dickinson
	FITC	HI30	BioLegend
CD64	BUV563	10.1	Becton Dickinson
	PerCP-Cy5.5	10.1	BioLegend
CD103	BV421	Ber-ACT8	BioLegend
CD172a (SIRPα)	PerCP eFluor 710	P84	Invitrogen
	PE-Cy7	SE5A5	BioLegend
CD183 (CXCR3)	BUV737	1C6/CXCR3	Becton Dickinson
CD282 (TLR2)	BUV615	11G7	Becton Dickinson
FOXP3	FITC	PCH101	Invitrogen
HLA-DR	BV510	L243	BioLegend
IFN	APC	4S.B3	BioLegend
IL-1β	PE	AS10	BD Fastimmune
IL-6	PE Cy7	MQ2-13A5	BioLegend
IL-10	BV711	JES3-9D7	Becton Dickinson
	PEDazzle 594	JES3-9D7	BioLegend
IL-15	Alexa Fluor 594	34559	R&D systems
IL-17a	APC-Cy7	BL168	BioLegend

Antigen Specificity	Conjugate	Clone	Manufacturer
JAK1	Alexa Fluor 647	413104	R&D systems
JAK3	Alexa Fluor 488	452524	R&D systems
RORγt	PE	B2D	Invitrogen
STAT5 (Phospho Tyr694)	PE	A17016	BioLegend
STAT6 (Phospho Tyr641)	Alexa Fluor 488	A15137E	BioLegend
T-bet	PE-Cy7	4B10	BioLegend
TLR4	BV605	TF901	Becton Dickinson
TNFα	BV785	MAb11	BioLegend

6.2.6 Flow cytometry analysis

In all cases, cells were acquired on a Cytek Aurora (5 laser) cytometer (Cytek, California, USA) and analysed using OMIQ Data Science platform (© Omiq, Inc. 2022).

For the supervised analysis, total HLA-DR$^+$ cells were identified withing singlet viable CD45$^+$ cells and categorized into conventional dendritic cells (cDC), monocytes and Mϕ based on the expression levels of CD14 and CD11c as shown in Figure 6.1. Further quantification of the expression levels of each marker was determined using the fluorescence minus one approach as shown in Figure 6.2.

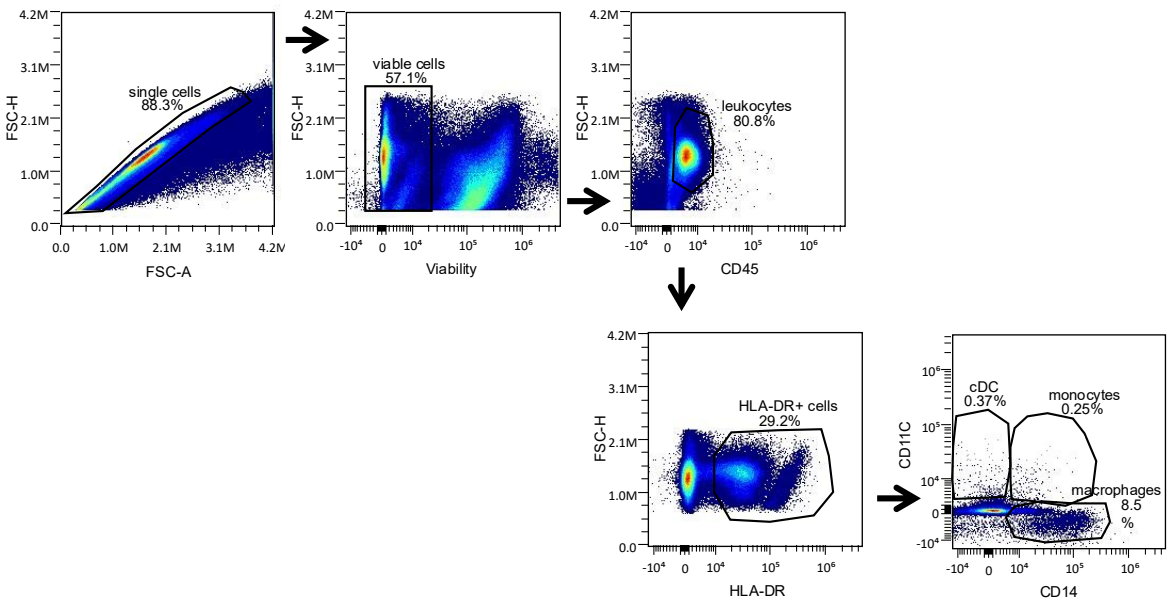

Figure 6.1. Human intestinal conventional dendritic cells, monocytes and macrophages.
Human intestinal antigen presenting cells, including conventional dendritic cells (cDC, CD14-CD11c+), monocytes (CD14+CD11c+) and macrophages (CD14+CD11c-) were identified within single viable HLA-DR+ leucocytes in the human intestinal lamina propria. Results are representative of several independent experiments.

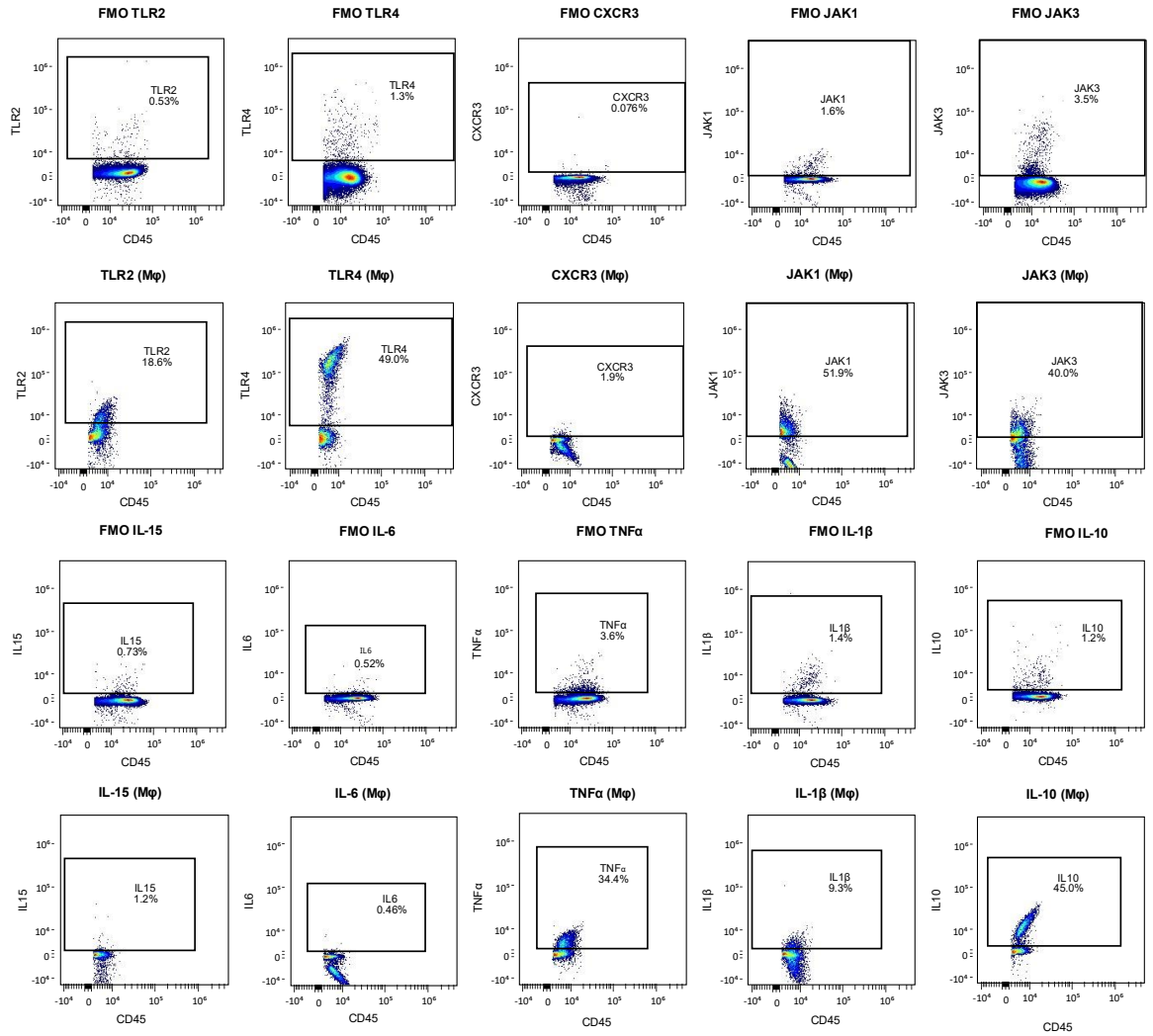

Figure 6.2. Characterization of human intestinal antigen presenting cells.
Human intestinal antigen presenting cells, including conventional dendritic cells (cDC), monocytes and macrophages (MΦ) were identified as in Figure 6.1. Expression levels of the different makers displayed in the figure were referred to their respective fluorescence minus one (FMO). Although the analysis is only shown for MΦ, the same approach was followed for cDC and monocytes.

For the unsupervised analysis, a complementary gating strategy was applied to select all APC (monocytes, macrophages and cDC) within single viable HLA-DR^{+}CD45^{+} following exclusion of CD14^{-}CD11c^{-} cells as shown in Figure 6.3. Building from that, an unsupervised approach applying Uniform Manifold Approximation and Projection (UMAP) algorithm was used. Subsequent FlowSOM algorithm was used to find similar cell subsets and separate them into groups in an unsupervised manner. A clustered heatmap was then created using the clusters obtained in the previous point. The refine results of FlowSOM algorithm were mapped on the UMAP to observe their distribution. Finally, Volcano plots were constructed with the edgeR algorithm comparing cluster differences.

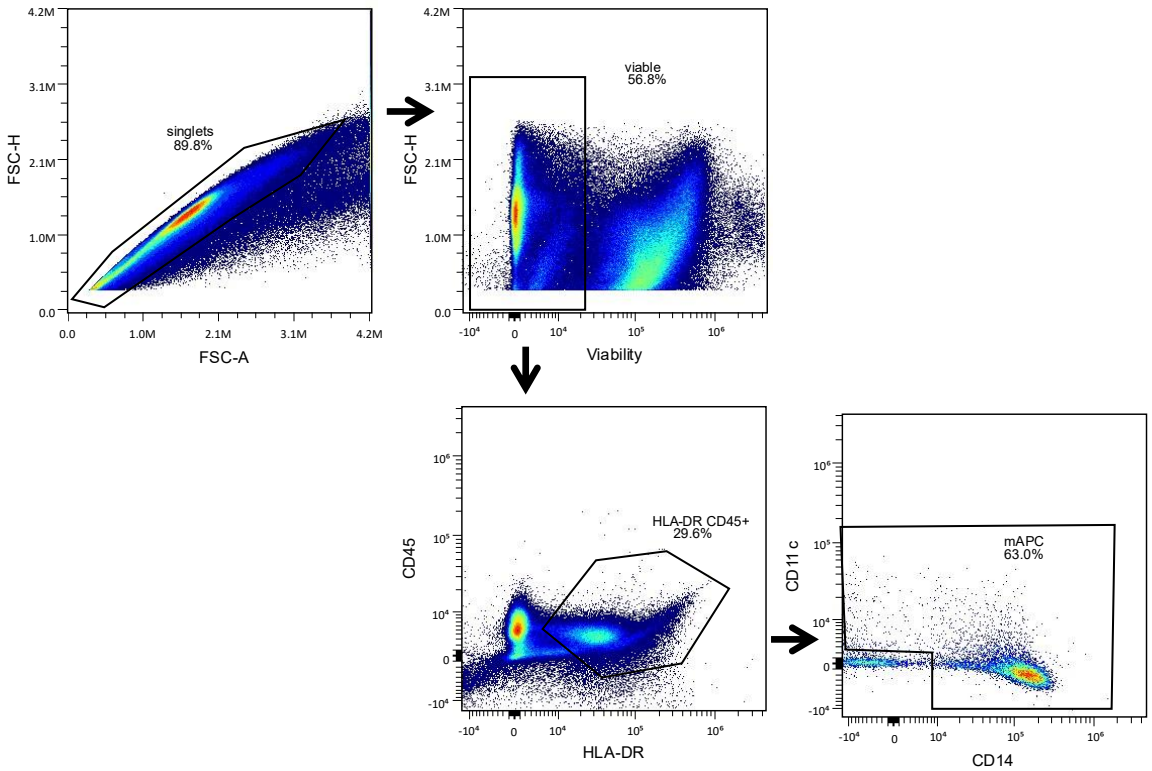

Figure 6.3. Total human intestinal myeloid antigen presenting cells identification.
Total human intestinal myeloid antigen presenting cells (mAPC) were identified within singlet viable HLA-DR$^+$ leucocytes discarding CD14$^-$CD11c$^-$ cells.

6.2.7 Statistical analysis

For statistical analysis, GraphPad Prism 9 was used for the supervised analysis. One-Way ANOVA, and t-test comparisons were also applied considering p-values <0.05 significant. As for the flow cytometry unsupervised analysis, volcano plots were constructed with the edgeR algorithm comparing cluster differences in the unsupervised analysis. In all cases, a p-value under 0.05 were considered statistically significant.

6.3 RESULTS

6.3.1 Unsupervised characterization of human colonic APC in health and UC.

First, total human intestinal APC from the controls, as well as from patients with active and quiescent UC were identified as in Figure 6.3. The UMAP analysis identified 4 major islands (Figure 6.4A). The relative contribution of each marker on the UMAP structure is shown in Figure 6.4B.

Given that CD11c can be used as a surrogate marker to discriminate human intestinal monocytes ($CD11c^+$) and Mϕ ($CD11c^-$) (15), monocytes and cDC seem to be restricted to island found on the top at the right as it is $CD11c^+$. Indeed, cDC seem to be found on the upper side of such island as cells are $CD14^-$ and express CD103, JAK1 and TLR2. On the contrary, the lower side of such island seem to be $CD14^+$ inactivated monocytes. On the other hand, both islands on the left seem to be Mϕ ($CD11c^-$). Although the top one express higher CD14 expression, both express TLR4, JAK3 and IL-1β. On the other hand, the lower island on the right seems to be non-activated $JAK1^+$ macrophages which do not express TLR4 and have lower production of IL-1β.

To further refine our analysis, the FlowSOM algorithm was used to find similar cell subsets and separate them into clusters in an unsupervised manner. A total of 16 clusters were identified according to the expression of the different markers as shown in the heatmap (Figure 6.4C), which also reveals a close relationship between the cells from patients with quiescent UC and controls, referred to patients with active disease. These clusters were further plotted in the UMAP to relate one with each other (Figure 6.4D).

Volcano plots revealed that 6 of the clusters were significantly increased in controls when compared with aUC, and only one was increased in aUC. When comparing aUC and qUC, 6 of the clusters were significantly increased in qUC and 2 of them were decreased. Finally, only 1 cluster was differentially represented between controls and patients with qUC (Figure 6.4E).

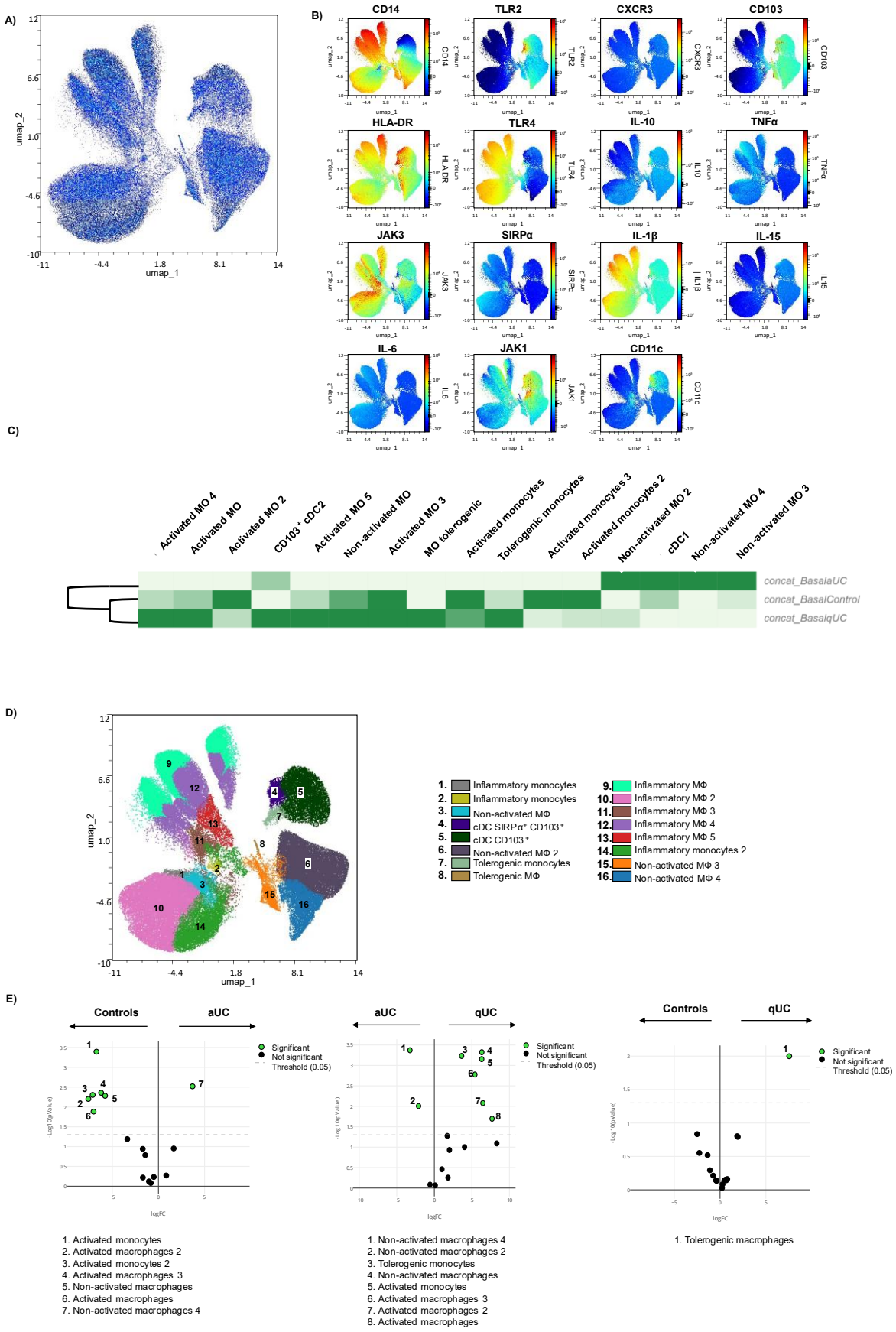

Figure 6.4. Unsupervised analysis of human intestinal antigen presenting cells.
A) *Total myeloid antigen presenting cells (mAPC) were identified within singlet viable leukocytes as in Figure 6.3,and analyzed with a Uniform Manifold Approximation and Projection (UMAP) on resting conditions (n=30).*

B) *Expression intensities of the analyzed markers represented with a color code based on the intensity where red represents higher expression and blue, lower expression.* ***C)*** *Heatmap displaying the intensity levels of each identified cluster within the three cohorts.* ***D)*** *All 16 clusters were overlaid on the UMAP projection using a specific color and number as shown in the legend.* ***E)*** *Volcano plots comparing the different clusters among the 3 study groups highlighting in green those with statistically significant differences.*

6.3.2 Tofacitinib induced a specific reduction of phosphorylated STAT5.

In order to confirm the inhibitory capacity of Tofacitinib in our *ex-vivo model*, total PBMC were overnight cultured in the presence of different concentrations (1, 10, 100 and 1000 nM) of Tofacitinib citrate and in basal conditions (no stimulus) before addressing phosphorylated STAT5 and STAT6 intracellular content. Results revealed that Tofacitinib induced a specific reduction of phosphorylated STAT5 but not STAT6 (Figure 6.5).

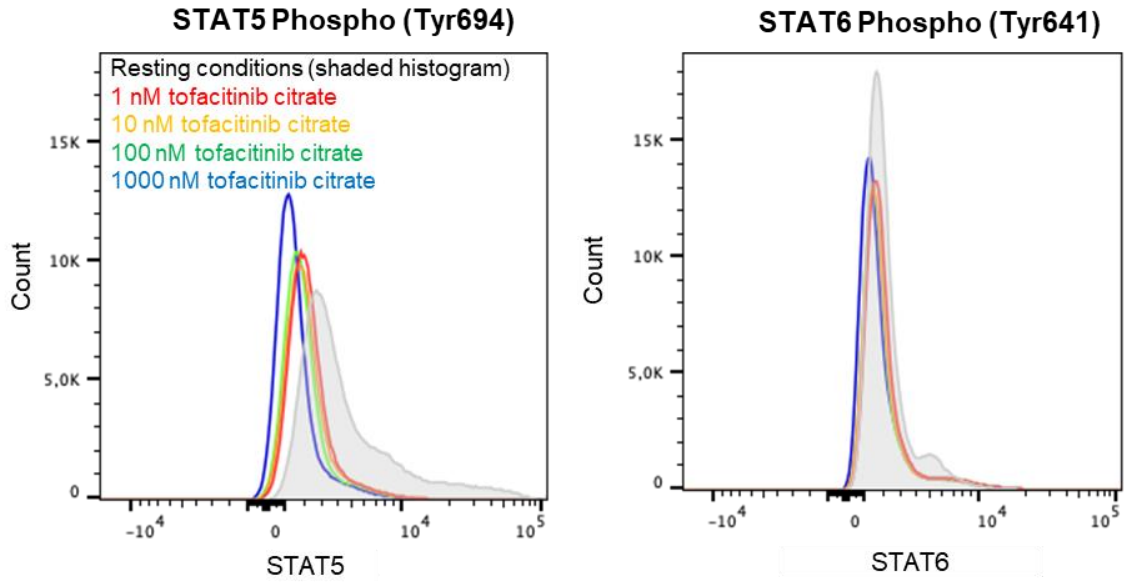

Figure 6.5. Intracellular phosphorylated STAT content.
Total peripheral blood mononuclear cells were overnight cultured in the presence of different concentrations (1, 10, 100 and 1000 nM) of Tofacitinib citrate before addressing phosphorylated STAT5 and STAT6 intracellular content referred to resting conditions (shaded histogram). Results are representative from several independent experiments.

6.3.3 Tofacitinib JAK1 down regulation is restricted to intestinal monocytes.

Having demonstrate the modulatory capacity of tofacitinib over PBMC, we next assessed the effect that Tofacitinib elicits on LPMC following LPS stimulation. Our results revealed that Tofacitinib decreased IL-6 production, as well as the expression of TLR4 and JAK3 on macrophages. On the other hand, Tofacitinib decreased JAK3 levels on monocytes while it restored CXCR3 downregulation caused by LPS on cDC (Figure 6.6).

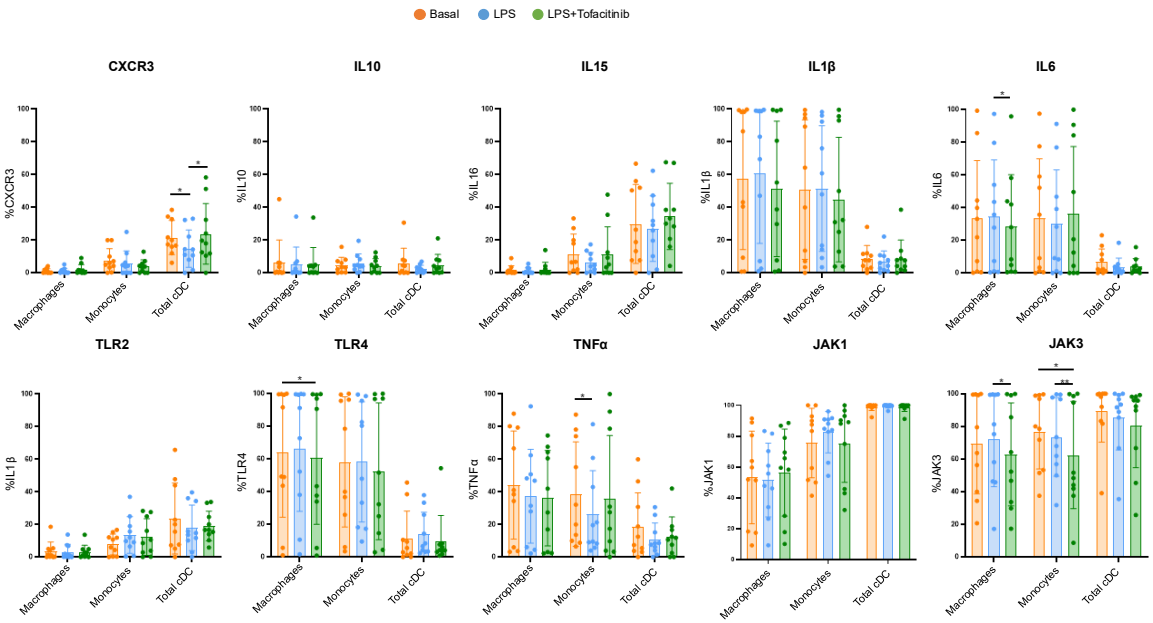

Figure 6.6. Tofacitinib effect over human intestinal antigen presenting cells.
*Total lamina propria mononuclear cells (LPMC) from controls, were ex-vivo cultured in resting conditions (Basal), as well as with 100ng/ml of LPS in the presence/absence of 100nM Tofacitinib. Total monocytes, macrophages and conventional dendritic cells (cDC) were identified as in Figure 6.1, as assessed for the expression of CXCR3, IL-10, IL-15, IL-16, IL-6, TLR2, TLR4, TNFα, JAK1 and JAK3 based on their respective fluorescence minus one (FMO) controls as in Figure 6.2. Two-way ANOVA was applied. P-values <0.05 were considered significant (*p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001).*

When focused on patients with active UC, our results shown that only JAK1 expression on both macrophages and monocytes, but not cDC, were decreased following Tofacitinib stimulation (Figure 6.7). Similar observations were found in the case of patients with quiescent disease, where Tofacitinib decreased JAK1 and JAK3 levels, as well as increased TLR2 levels on monocytes, and decreased IL-15 production by cDC had no effect on macrophages (Figure 6.8).

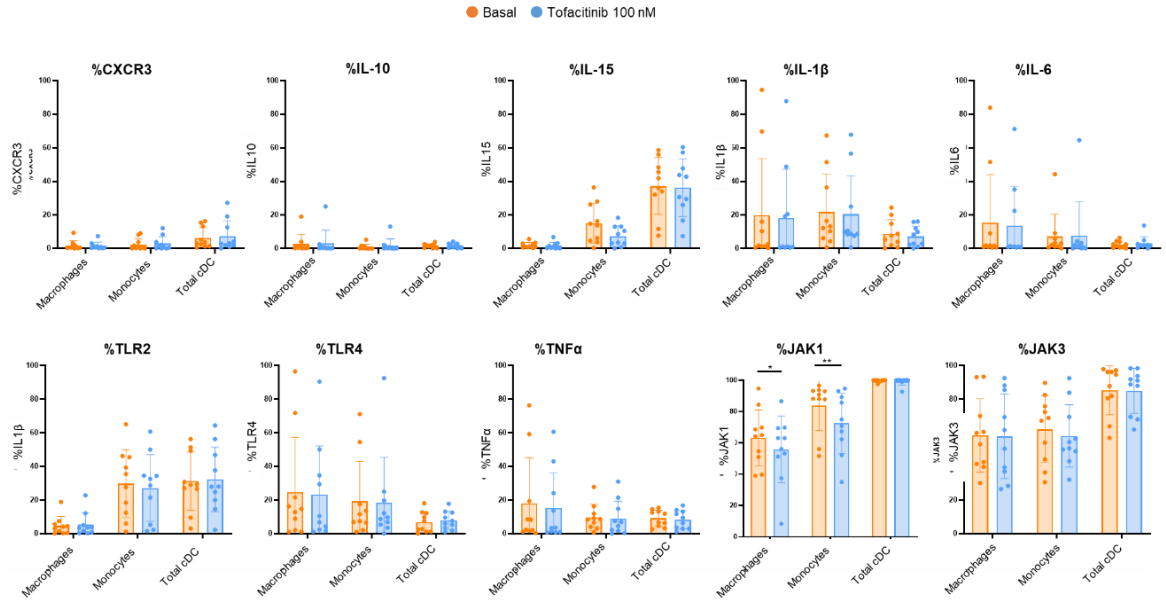

Figure 6.7. Tofacitinib modulation of human intestinal antigen presenting cells from patients with active ulcerative colitis.
*Total lamina propria mononuclear cells (LPMC) from patients with active ulcerative colitis were ex-vivo cultured in resting conditions (Basal) as well as with 100nM Tofacitinib. Subsequent expressions of CXCR3, IL-10, IL-15, IL-1β, IL-6, TLR2, TLR4, TNFα, JAK1 and JAK3 on monocytes, macrophages and conventional dendritic cells (cDC) was determined as in Figure 6.6. Two-way ANOVA was applied. P-values <0.05 were considered significant (*p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001).*

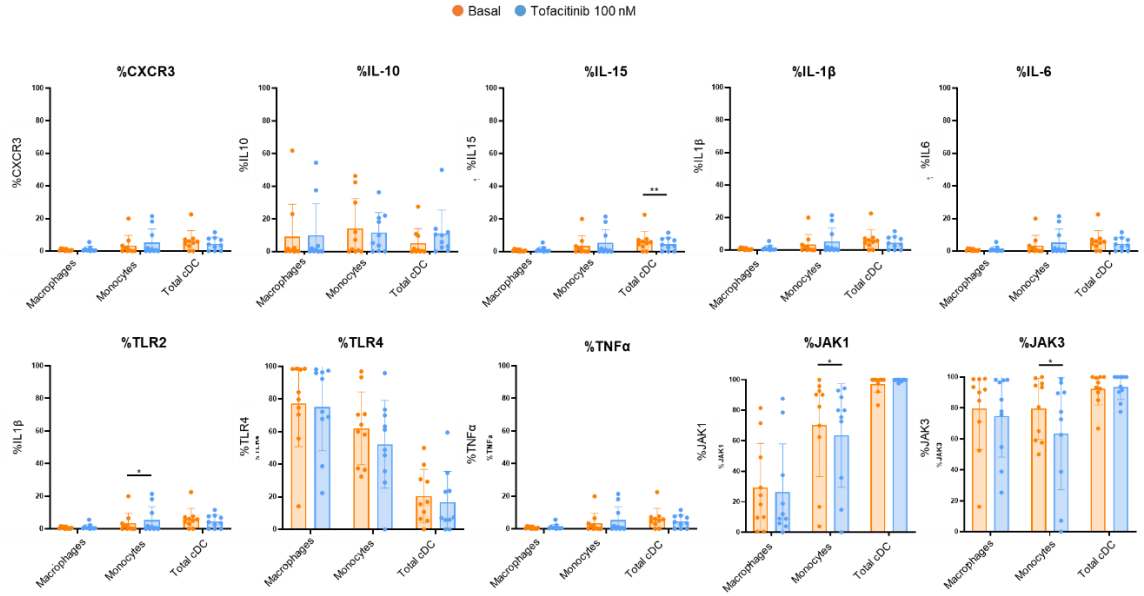

Figure 6.8. Tofacitinib effect over human intestinal antigen presenting cells from patients with quiescent ulcerative colitis.
*Total lamina propria mononuclear cells (LPMC) from patients with quiescent ulcerative colitis were ex-vivo cultured in resting conditions (Basal) as well as with 100nM Tofacitinib. Subsequent expressions of CXCR3, IL-10, IL-15, IL-1β, IL-6, TLR2, TLR4, TNFα, JAK1 and JAK3 on monocytes, macrophages and conventional dendritic cells (cDC) was determined as in Figure 6.6. Two-way ANOVA was applied. P-values <0.05 were considered significant (*p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001).*

6.3.4 Tofacitinib does not modulate the outcome of human intestinal cDC

Although Tofacitinib did not elicit any major effect of the phenotype of human intestinal cDC from patients with UC (either active or quiescent), we finally addressed whether it could, modulate cDC function given their central role at driving disease inflammation in UC (1–3,16). Given that UC patients, as opposed to those with CD, hardly have tissue resections, we used a model of controls using human intestinal cDC sorted from human resection which had been previously activated with/out LPS.

Human intestinal cDC induced naïve T cell proliferation (Figure 6.9) as opposed to the monocytes/macrophages counterparts (data not shown). Overall, our results revealed that Tofacitinib decreased the stimulatory capacity of LPS-activated colonic cDC but not their ileal counterparts (Figure 6.10A). Of note, when further analysis was performed to determine whether such reduction was mainly elicited on the helper or the cytotoxic fraction, no differences were found (Figure 6.10B). Finally, we also assessed the profile of the stimulated T cells in order to determine whether Tofacitinib could modulate the outcome of the T cells responses towards Treg (FOXP3, IL-10), Th1 (Tbet, IFNY) or Th17 (RORγt, IL-17) which was not the case (Figure 6.10C) therefore confirming that Tofacitinib does not modulate the phenotype and function of human intestinal cDC.

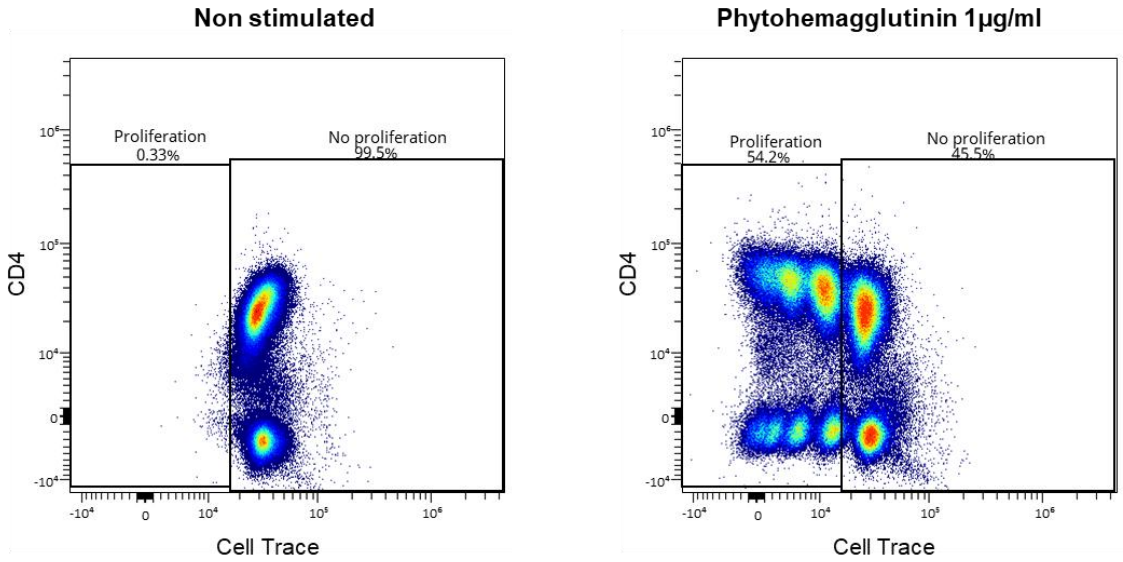

Figure 6.9. T cell proliferation.
CellTrace™ Violet naïve T cells were cultured for 5 days in resting condition or with 1µg/ml of phytohemagglutinin. Total T cells were identified within singlet viable CD3⁺ and those divided cells identified by CellTrace™ Violet dilution.

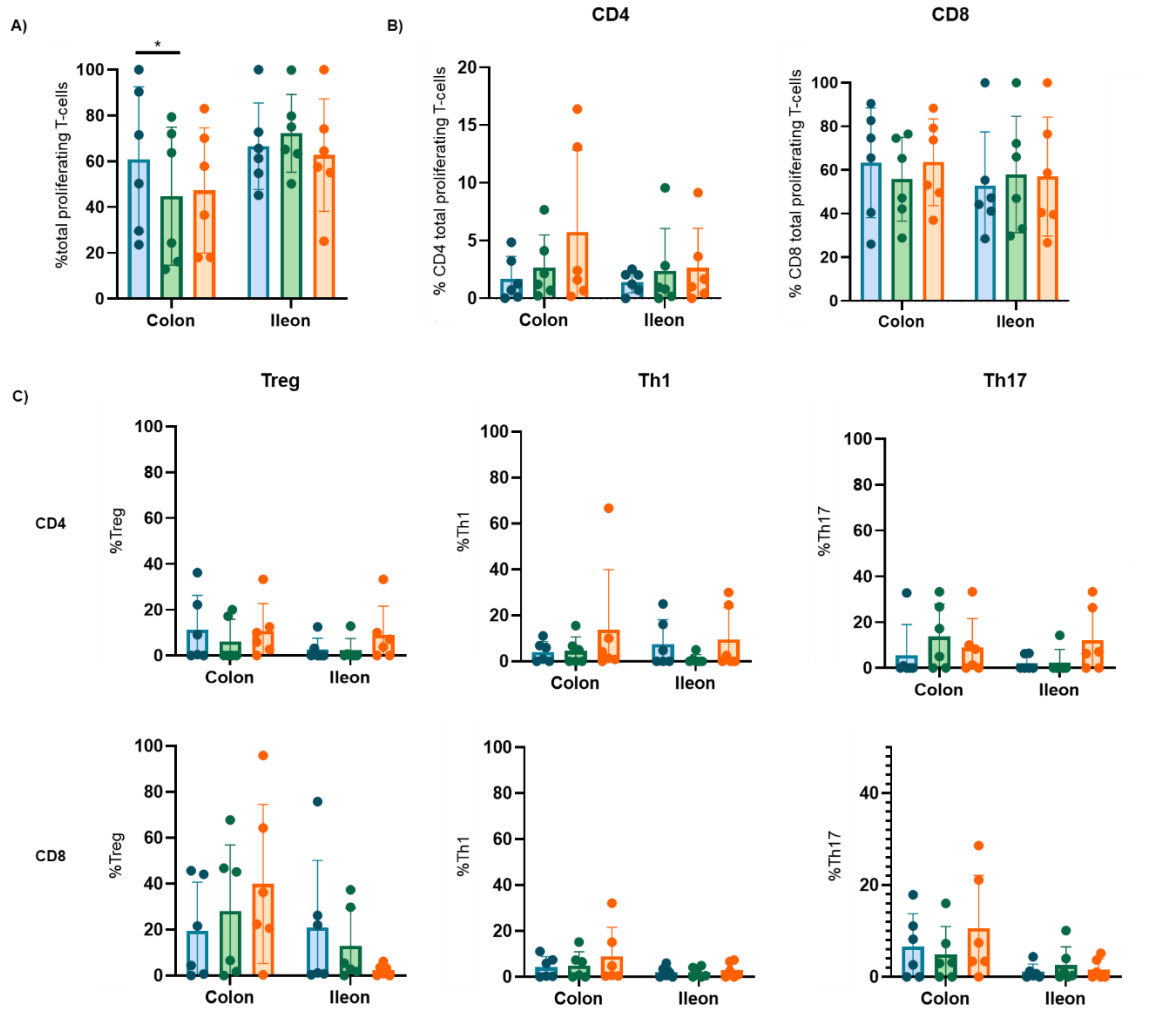

Figure 6.10. Tofacitinib effect over the immunostimulatory capacity of human intestinal conventional dendritic cells.

A) *Total lamina propria mononuclear cells were ex-vivo cultured in resting conditions (Basal), as well as with 100 ng/ml of LPS in the presence/absence of 100 nM Tofacitinib. Total conventional dendritic cells (cDC) were subsequently sorted and co-cultured with allogeneic cell-trace violet labelled naïve T cells. cDC stimulatory capacity over total T cells was determined, as well as* ***B)*** *the stimulatory capacity specifically elicited over both CD4 and CD8. The acquired phenotype (Treg: FOXP3$^+$IL-10$^+$; Th1: Tbet$^+$IFNγ^+; Th17: RORγt$^+$IL17$^+$) is displayed in* ***C)****. Two-way ANOVA was applied in all cases. P-values <0.05 were considered significant (*p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001).*

6.4 DISCUSSION

Although Tofacitinib inhibits the JAK1 and JAK3 signalling pathway (9), and its use has been approved to treat patients with UC (6–8), its specific mechanism of action (i.e. its main target cell) remains elusive. Given the central role that human intestinal cDC elicit controlling the outcome of the immune responses in health (17), and since their phenotype and function is altered in IBD, including UC (18,19), our hypothesis was that Tofacitinib would specifically modulate their phenotype and function. In order to address such hypothesis, we assessed the effect of Tofacitinib on human intestinal cDC, both in health and UC, referred to human intestinal monocytes and macrophages. Nevertheless, and contrary to our expectations, we found that Tofacitinib downregulates JAK1 expression on human intestinal monocytes (and to a lower extent JAK1 and JAK3 on macrophages) without affecting human intestinal cDC phenotype or function.

Previous studies have reported that Tofacitinib decreased human monocyte-derived dendritic cell (MoDC) stimulatory capacity (20) and differentiation (21). Nevertheless, the same has not been mirrored on human intestinal cDC. The origin of such discrepancy can be due to several reasons. The most obvious one is that those experiments were performed on LPS-activated MoDC which, nevertheless, do not resemble the properties of real cDC (22). Besides, we hereby have performed the experiments on real human intestinal cDC so although we cannot discard that Tofacitinib may modulate the phenotype and function of circulating cDC (either in health or UC), the same does not seem to be true in the human intestinal mucosa as we have observed. In a similar manner, our observations suggest that although cDC are central to in controlling the outcome of the human adaptive immune responses, once the pro-inflammatory Th1/Th17 signalling pathway has been triggered on the UC intestine (23)Tofacitinib does not modulate the outcome of cDC. On the contrary, its therapeutic effect may be suppressing pro-inflammatory T cell activation in the human gut (24,25) without modulating cDC.

In order to further confirm the inhibitory effect of Tofacitinib in our ex-vivo model, in addition to specifically address the intracellular expression of JAK1 and JAK3, further downstream analysis revealed that Tofacitinib induced a specific reduction of phosphorylated STAT5 (but not STAT6) which confirms the Tofacitinib-induced JAK inhibition (Figure 6.5). Building from that, we hereby have found that although Tofacitinib did not seem to elicit any major effect on human intestinal cDC, it actually downregulated JAK1 on human intestinal monocytes from UC (both active and quiescent). On the other hand, JAK1 was decreased on macrophages from patients with active UC, while JAK3 was downregulated on patients with quiescent disease following Tofacitinib exposure. Previous observations have shown

that Tofacitinib induces a regulatory phenotype on human primary monocytes and monocyte-derived macrophages (26–29). Building from that, and as opposed to the previous observations on the cDC context, we hereby have confirmed, for the first time to our knowledge, that Tofacitinib downregulates JAK1 and JAK3 on *bona fide* human intestinal monocytes and macrophages.

We have also performed an unbiased characterization (in resting conditions) of the total mAPC subsets than can be found in the human colon in health and UC, both active and quiescent, by spectral and computational cytometry (30). Our results have found that, based on the markers that we have used, we can identify up to 16 different subsets of human intestinal mAPC, being most of them macrophages in agreement with previous observations from our group (15). Besides, and as expected, mAPC from patients with qUC were more similar to those from controls rather than to those from UC patients with active mucosal inflammation. Nevertheless, when the same approach was performed to assess whether Tofacitinib would expand or decrease a given cell cluster in any of the patients, no differences were found. Hence, these results imply that although Tofacitinib downregulated the JAK1, and to a lesser extent, the JAK3 signalling pathway on monocytes and macrophages, that is not translated into a major phenotype change on these cells. Nevertheless, whether that translates into a differential function on such cells remains elusive. Hence, a major limitation of this study is that we have focused specifically on the effect that Tofacitinib elicited over cDC rather that over monocytes or macrophages. Therefore, future studies should address whether Tofacitinib modulates monocytes and macrophage function or whether, on the contrary, its main mechanism of action is elicited over immune cells (like T cells as previously comments).

In summary, other previous models have suggested that Tofacitinib modifies the phenotype and function of human MoDC, we hereby have proved that human intestinal cDC are not modulated by such compound. These therefore has major implications given the differences between the mucosal and circulating immune system. Our results therefore highlight the relevance of performing experiments on the human intestinal mucosa if we want to unveil the mechanism of action of any drug in the IBD setting given the differences between the mucosal and the circulating immune system.

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Chapter 7. Characterization of the Gut Microbiota in Inflammatory Bowel Disease: Insights into Bacterial-Fungal Interactions and Potential Biomarkers

Manuscript sent for publication

7.1 INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic inflammation of the gastrointestinal (GI)-tract mediated by an altered immune response. IBD includes Crohn's disease (CD) and Ulcerative colitis (UC), which differ in their inflammatory patterns, intestinal location, clinical symptoms and associated complications (1,2). Although the aetiology of the disease is not yet known, IBD is widely considered to result from an exacerbated immune response against enteric microorganisms in genetically susceptible individuals (3). Its prevalence is increasing, especially in western countries, with an estimated 3.5 million cases of IBD in Europe and the United States (4).

The immune system maintains a delicate balance between immune responses against pathogens and tolerance toward commensal microbiota. (5). In addition to the microbial composition, microbial-derived metabolites play a crucial role in immune modulation. For instance, short chain fatty acids (SCFAs) such as butyrate, propionate and acetate produced by bacterial fermentation of dietary fiber, modulate histone acetylases and gene expression, influencing immune responses. Notably, IBD patients exhibit a reduced abundance of butyrate-producing bacteria, such as *Roseburia spp* (6) microbial metabolism of tryptophan generates bioactive metabolites containing indole, that affect the host by activating the aryl hydrocarbon receptor, a transcription factor present in immune cells that regulates inflammation, this pathway is impaired in IBD (7). Moreover, secondary BAs produced by bacteria modulate immune cells maturation and cytokines release (8), yet their production is decreased in patients with IBD.

The gut microbiota is influenced by host genetics, environmental factors, lifestyle and diet. The transition to a state of dysbiosis can trigger or exacerbate autoimmune and inflammatory responses (9–11). Amplicon sequencing of the 16S ribosomal RNA gene have revealed differences in the intestinal microbiota signature in IBD patients compared to healthy controls (12), including a depletion of *Firmicutes* (which have an anti-inflammatory effect) and *Clostridium* species*,* as well as an increase in the abundance of *Bacteroidetes* and *Proteobacteria,* such as *Bacteroides, Bifidobacterium* and *Lactobacillus,* which are associated with inflammation (7,13).

Other enteric microorganisms such as fungi and virus also contribute to gut eubiosis. Despite limited studies focused on elucidating the role that GI-tract fungi and viruses may have in the etiology of IBD, dysbiosis in these populations has been associated with the disease (14). IBD patients exhibit a higher *Basidiomycota/Ascomycota* ratio*,* reduced *Saccharomyces cerevisiae* and increased *Candida albicans* compared to healthy individuals (15). While numerous fungal species have been detected in the human gut, only

a few are commonly found across individuals. Meanwhile, the enteric virome is gaining scientific interest due to its potential impact on digestive tract homeostasis (16). However, the high variability observed among individuals complicates the identification of specific relationships IBD-virome (17,18).

Despite advances in understanding IBD, its pathogenesis remains incompletely defined, and current treatments are far from optimal. Therefore, a comprehensive characterization of enteric microorganisms in different IBD types (UC and CD) and disease states (active vs. quiescent) is needed. The present study aims to analyse the microbial composition of faeces and biopsies from IBD patients and healthy controls. Hence, these results might identify microbial interactions and their potential role in disease pathogenesis. This knowledge could contribute to improved diagnostic and therapeutic strategies.

7.2 MATERIAL AND METHODS

7.2.1 Patients and biological samples

Biopsies and stool samples were obtained from four groups of patients with IBD: Crohn's disease (CD) -both active and quiescent-, and ulcerative colitis (UC), also active and quiescent. Samples were also collected from individuals attending medical consultation for reasons other than IBD and who have healthy mucosa during colonoscopy and constitute control group. Samples were collected from both the Hospital Clínico Universitario de Valladolid and Hospital Universitario Río Hortega de Valladolid. All participants provided signed informed consent (approval code by the CEIm Area del Salud de Valladolid PI 22-2869). Information was collected for each patient regarding age and gender, current treatments, as well as any treatments received in the four weeks prior to sample collection (if applicable) including antibiotics (people who had taken antibiotics for at least three months prior to sample collection were discarded). Additionally, disease phenotype in CD (B1 inflammatory; B2 stenosing; B3 penetrating or fistulizing), disease location according to the Montreal classification: CD (L1 ileal; L2 colonic; L3 ileocolonic; L4 upper gastrointestinal tract), UC (E1 proctitis; E2 distal colitis; E3 extensive colitis) any other information that might be relevant for interpreting the results was also collected and it is shown in Table 7.1.

Faecal samples were obtained from 30 individuals: 5 controls (40% men, 61 ± 6 years), 6 patients with active Crohn’s disease (83% men, 48 ± 18), 6 patients with quiescent Crohn’s disease (71% men, 42 ± 12), 5 patients with active ulcerative colitis (60% men, 58 ± 12) and 7 patients with quiescent ulcerative colitis (57% men, 54 ± 9). Biopsies were obtained from the ileum of 24 of these 30 patients (5 from each group). Patient 11- from quiescent Crohn's cohort- was excluded from the study due to not meeting the inclusion criteria, as antibiotic use was identified.

Stool samples were collected in faecal collection tubes (Canvax Biotech, Valladolid, Spain) during the 24 hours prior to the endoscopy and cryopreserved at -80°C. Following biopsy obtention, they were preserved in ice-chilled Phosphate-buffered saline (PBS) (ThermoFisher Scientific, Waltham, USA) and subsequently cryopreserved at -80°C in RNAlater™ (ThermoFisher Scientific, Waltham, USA).

Table 7.1. Patient demographics.

Code	IBD type	IBD subtype	Gender	Age	UCEIS	SES-CD	Treatment
1*	Active colitis	N/A	Male	47	4		Mesalazine, Azatioprine and Infliximab
2	Control	N/A	Male	50			N/A
3	Control	N/A	Female	66			N/A
4	Quiescent colitis	N/A	Female	49	0		Mesalazine
5	Quiescent colitis	N/A	Male	57	1		Oral mesalazine
6	Quiescent colitis	N/A	Male	63	0		Azatioprine
7	Quiescent Crohn	Ileal L1	Male	57		0	N/A
8	Quiescent colitis	N/A	Male	62	0		Mesalazine
9*	Active colitis	N/A	Male	56	3		Azatioprine and oral+topic Mesalazine
10	Quiescent colitis	N/A	Female	43	0		Golimumab
11	Quiescent Crohn	Ileocolonic L3	Male	29		0	Ciprofloxacine and Metronidazol
12	Active Crohn	Ileocolonic L3	Male	41		9	Mesalazine
13	Quiescent colitis	N/A	Female	45	0		Infliximab and Mesalazine
14	Quiescent Crohn	Ileal L1	Female	43		0	Infliximab
15	Quiescent Crohn	Ileocolonic L3	Female	46		0	Metotrexate
16*	Quiescent Crohn	Colonic L2	Male	44	0		Azatioprine
17	Active colitis	N/A	Female	47	4		Vedolizumab
18	Active colitis	N/A	Male	73	3		Vedolizumab and oral Mesalazine
19	Control	N/A	Male	64			N/A
20	Control	N/A	Female	64			N/A
21	Control	N/A	Female	63			N/A
22	Quiescent Crohn		Male	24		0	Azatioprine
23	Quiescent Crohn		Male	52		0	Ustekinumab
24	Active colitis		Female	67	4		Vedolizumab and oral Mesalazine
25	Active Crohn	Ileal	Female	64			Azatioprine
26*	Active Crohn		Male	20		5	Ustekinumab
27	Active Crohn		Male	58		6	N/A
28*	Quiescent colitis	N/A	Male	62	0		Vedolizumab and oral Mesalazine
29	Active Crohn	Ileal	Male	40		3	Mercaptopurine
30	Active Crohn	Ileal	Male	66		3	Adalimumab

**Only faecal samples*.

7.2.2 Biopsies processing and sequencing

DNA extraction and sequencing was carried out on Seqplexing (Sequencing Multiplex, Valencia) using an Illumina MiSeq device, paired-end 2x250bp. For bacteria (16S DNA), the primers used for amplification were 16S V1-V2 Forward Primer 5'-1: TNANACATGCAAGTCGRRSG; 16S V1-V2 Forward Primer 5'-2: TAACACATGCAAGTCRACTYGA and 16S V1-V2 Reverse Primer 3': GCTGCCTCCCGTAGGAGT. For fungi (ITS2 region), the primers used were ITS2_F1: GTGARTCATCGAATCTTTG, ITS2_R1: TCCTCCGCTTATTGATATGC and ITS2_R2: GATATGCTTAAGTTCAGCGGGT. Biopsies were also used to study viral composition using a *Shotgun* approach also in Seqplexing (Sequencing Multiplex, Valencia).

7.2.3 Stool samples processing and sequencing

Bacterial DNA was extracted following manufacturer's recommendations for the QIAmp PowerFaecal Pro DNA Kit (Qiagen, Venlo, Netherlands). DNA was quantified using a NanoDrop microvolume spectrophotometer (Thermo Fisher, USA) and then kept at -20°C until shipment. Metagenomic sequencing was performed at Agricultural Technology Institute of Castilla y León (ITACYL) in Myseq platform (Illumina, San Diego, CA, USA) and using the Nextera XT Index Kit paired-end (2x300nt) (Illumina, San Diego, CA, USA) for microbiota analysis by amplifying the hypervariable V3 and V4 regions of 16S rDNA with specific primers (515F-806R).

7.2.4 Bioinformatic analysis.

Data analysis was firstly performed using Qiime2 (https://qiime2.org/), an open-source software, to obtain the necessary datasets to perform the further analysis using R (https://www.r-project.org/). All the code related to this project is available in the following GitHub repository: https://github.com/mariagpms/Microbiome-Analysis.git

7.3 RESULTS

7.3.1 Microbial diversity in intestinal biopsies

7.3.1.1 Bacterial diversity

Alpha diversity in intestinal biopsies was analysed using both Shannon and Simpson indices to assess bacterial diversity (Figure 7.1). Even though there were no significant differences, alpha diversity indices revealed distinct microbial patterns. The control group exhibited a well-balanced microbial diversity with a slight predominance of certain species. In contrast, both active UC and CD groups showed increased microbial diversity but with a lower dominance of specific taxa. Notably, the quiescent UC and CD groups displayed the highest diversity and evenness, suggesting a partial restoration of microbiota composition during remission (Figure 7.1).

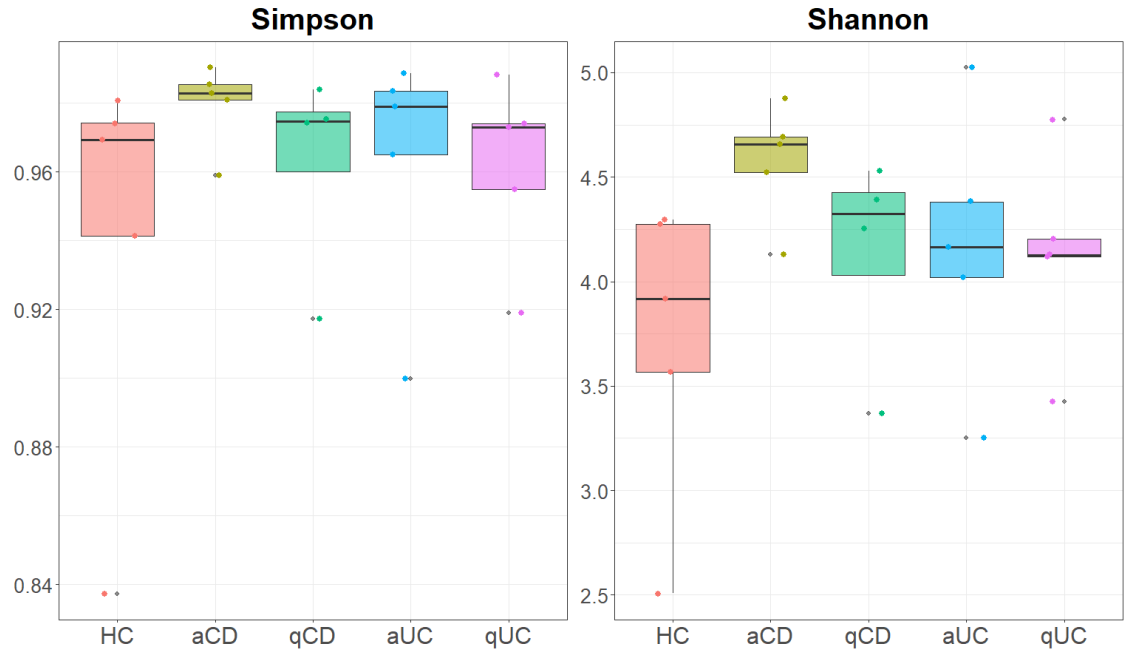


Figure 7.1. Bacterial alpha diversity in intestinal biopsies.
Shannon and Simpson indices of alpha diversity of the bacteria were calculated in intestinal biopsies from the different studied groups: healthy controls (HC), active Crohn's disease (aCD), quiescent Crohn's disease (qCD), active colitis (aUC) and quiescent colitis (qUC). Kruskal Wallis test was then performed. P-value < 0.05 was considered significant.

Beta diversity analysis revealed no significant differences between groups. Bray-Curtis, Jaccard, and UniFrac (weighted and unweighted) distance metrics were used to assess community composition. Although no statistically significant differences were detected, variability among individuals was evident, suggesting a heterogeneous microbial landscape irrespective of disease status (Figure 7.2).

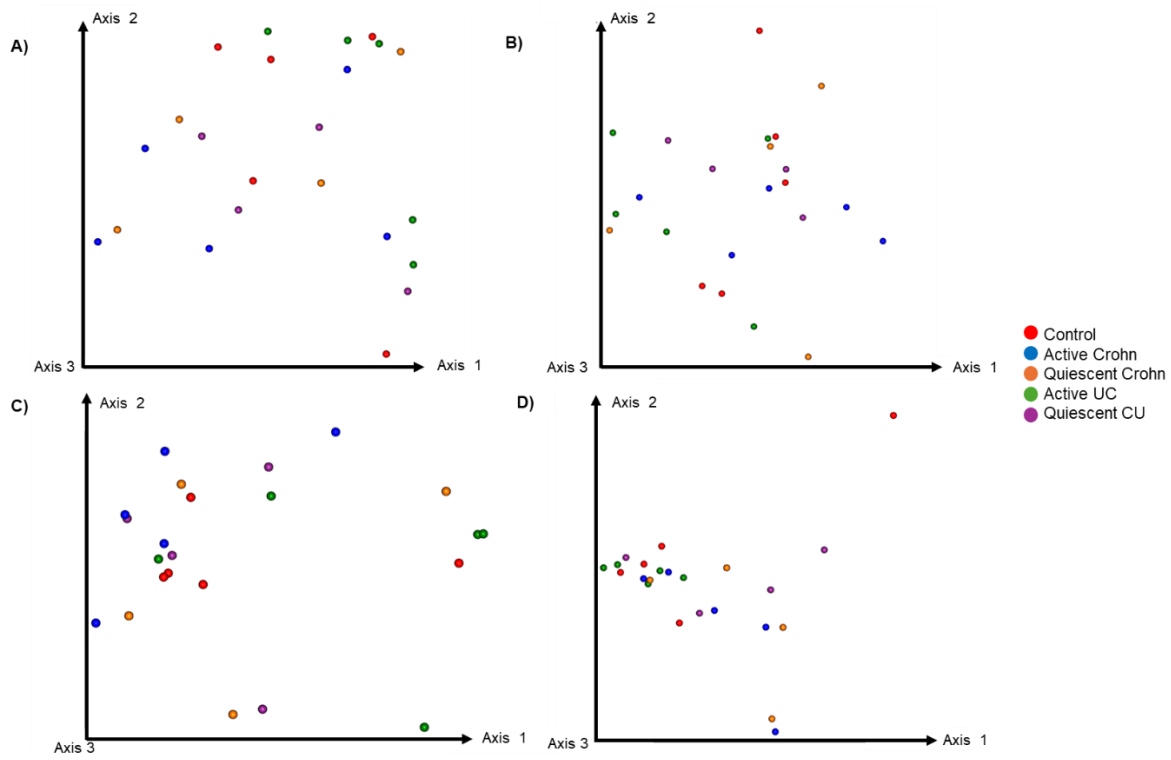


Figure 7.2. Bacterial beta diversity in intestinal biopsies.
*PCoA showing beta diversity was calculated using different distances: **A)** Bray Curtis, **B)** Jaccard, **C)** Unweighted Unifrac, **D)** Weighted Unifrac.*

7.3.1.2 Bacterial composition

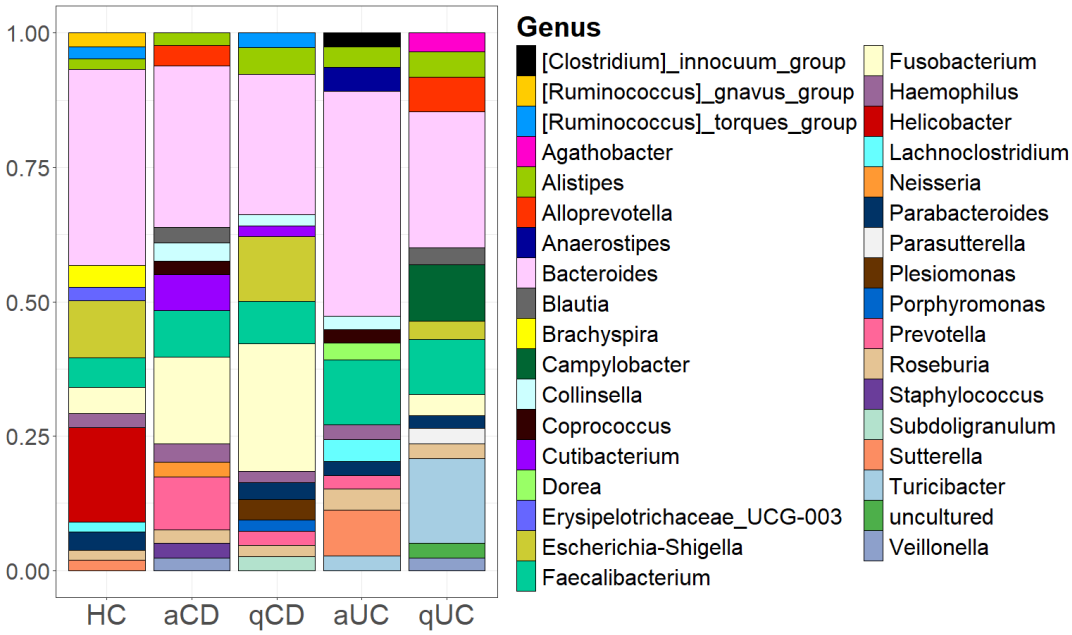


Figure 7.3. Top 15 genus of bacteria in biopsies.
The 15 most abundant bacteria genera found in intestinal biopsies were identified for each group: healthy controls (HC), active Crohn's disease (aCD), quiescent Crohn's disease (qCD), active colitis (aUC) and quiescent colitis (qUC). Relative frequencies of each genus were calculated and those which relative frequencies were not assigned to any genus (N/A) were discarded. Values of these 15 genera were standardized to 0-1 to calculate the percentage that is represented.

Distinct bacterial signatures were observed among groups. In control group, *Bacteroides, Escherichia-Shigella* and *Helicobacter* were predominant. *Helicobacter* was associated with a single infected patient and excluded from further taxonomic analysis. Interestingly, *Brachyspira*, *Erysipelotrichaceae*, and *Ruminococcus gnavus* were detected exclusively in control samples. Active CD patients exhibited a high abundance of *Bacteroides*, *Fusobacterium, Prevotella* and *Faecalebacterium*, while in quiescent CD patients, the most abundant genera were *Bacteroides*, *Fusobacterium* and *Escherichia-Shigella*. Notably *Neisseria* and *Staphylococcus* were exclusive to active CD, while *Porphyromonas*, *Plesiomonas* and *Subdoligranulum* were specific to quiescent CD. *Cutibacterium* was detected in both CD groups, but absent in UC and controls, suggesting its potential association with CD. In patients with active UC the most abundant genera were *Bacteroides, Faecalibacterium* and *Sutterella* while *Clostridium innocuum*, *Dorea*, and *Anaerostipes* were uniquely present in this cohort. Quiescent UC patients showed higher abundance of *Bacteroides*, *Turicibacter*, *Faecalebacterium* and *Campylobacter. Agathobacter, Campylobacter* and *Parasutterella* were found exclusively in this group. *Turicibacter* was specific to UC and in the same way, *Coprococcus* was identified only in active disease states (both UC and CD) (Figure 7.3).

To further investigate IBD-associated microbial changes, we compared relative abundance in IBD groups versus controls. In active CD *Fusobacterium* and *Faecalebacterium* were enriched, while in quiescent CD, *Fusobacterium* and *Faecalebacterium* and *Alistipes* were augmented. In active UC, there was an increase in *Bacteroides*, *Faecalebacterium, Lachnoclostridium, Roseburia* and *Sutterella.* Lastly, in quiescent UC *Alistipes* and *Faecalebacterium* were augmented together with a reduction in *Escherichia-Shigella* compared to controls.

A comprehensive summary of bacterial alterations is described in Table 7.6.

7.3.1.3 Fungi diversity

Bacterial microbiota has been extensively studied in IBD, leading to the validation of previously described findings. However, mycobiome remains less explored, despite its potential role in IBD. Alpha diversity was analysed using both Shannon and Simpson indices (Figure 7.4). Kruskal Wallis test was also performed and no significative differences among groups were observed (Shannon or Simpson index).

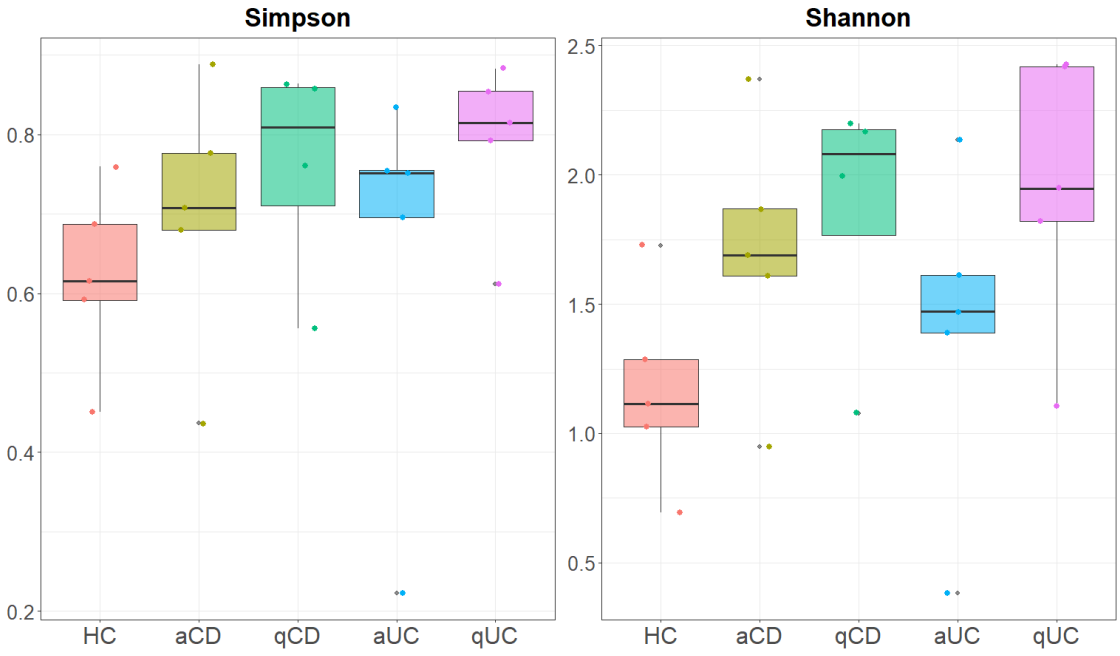


Figure 7.4. Fungal alpha diversity in intestinal biopsies.
Shannon and Simpson indices of alpha diversity of fungus were calculated in intestinal biopsies from the different studied groups: healthy controls (HC), active Crohn's disease (aCD), quiescent Crohn's disease (qCD), active colitis (aUC) and quiescent colitis (qUC). Kruskal Wallis test was then performed. P-value < 0.05 was considered significant.

The control group exhibited the lowest fungal diversity, with a moderate number of species and a clear dominance of a few taxa. Compared to controls, IBD patients with active disease (both UC and CD) showed increased mucosal fungal diversity, although a few species remained predominant. Notably, the highest fungal diversity was observed in quiescent IBD patients, suggesting a shift towards a more complex yet still unevenly distributed fungal community during remission.

Beta diversity analysis showed no significant differences among groups. Bray-Curtis, Jaccard, and UniFrac (both weighted and unweighted) distances were assessed (Figure 7.5).

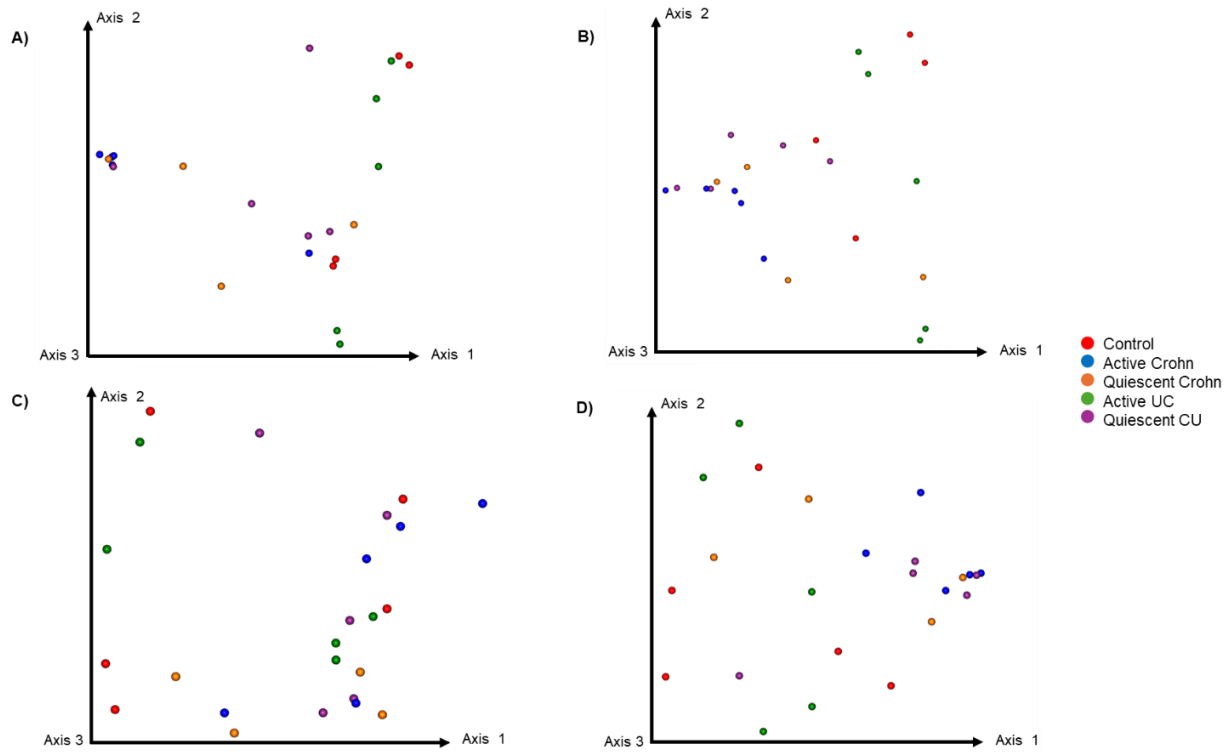


Figure 7.5. Fungal beta diversity in intestinal biopsies.
PCoA showing beta diversity was calculated using different distances: ***A)*** *Bray Curtis,* ***B)*** *Jaccard,* ***C)*** *Unweighted Unifrac,* ***D)*** *Weighted Unifrac.*

7.3.1.4 Fungi composition

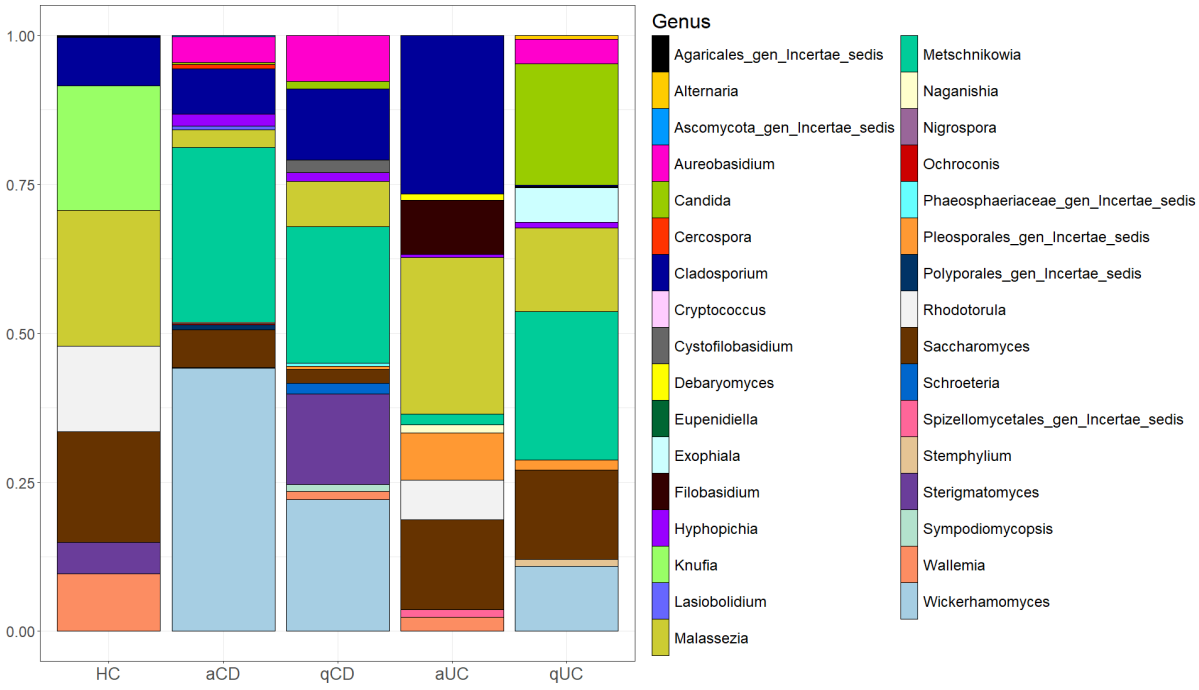


Figure 7.6. Top 15 genus of fungi in intestinal biopsies.
The 15 most abundant fungi genera found in intestinal biopsies were identified for each group: healthy controls (HC), active Crohn's disease (aCD), quiescent Crohn's disease (qCD), active colitis (aUC) and quiescent colitis (qUC). Relative frequencies of each genus were calculated and those which relative frequencies were not assigned to any genus (N/A) were discarded. Values of these 15 genera were standardized to 0-1 to calculate the percentage that is represented.

In control samples, a lower diversity of fungal genera was observed compared to IBD cohorts, with only seven genera represented, while IBD groups exhibited between 12 and 15 genera. The genera detected in controls included *Cladosporium, Candida, Malassezia, Rhodotorula, Saccharomyces, Sterigmatomyces, and Wallemia*; however, none were exclusive to this group. Distinct fungal signatures were observed across disease groups. In active CD, *Metschnikowia* and *Wickerhamomyces* were predominant, while *Cercospora, Lasiobolidium*, and *Polysporales* were exclusive to this cohort. Similarly, in quiescent CD, *Metschnikowia* and *Wickerhamomyces* remained the most abundant, with *Cystofilobasidium*, *Phaeosphaeriaceae*, *Schroeteria*, and *Sympodiomycopsis* being specific to this group. In active UC, *Cladosporium* and *Malassezia* were the most abundant genera, with *Debaryomyces*, *Filobasidium*, *Naganishia*, and *Spizellomycetales* uniquely present in this cohort. In quiescent UC, *Metschnikowia* and *Malassezia* were predominant, while *Alternaria*, *Exophiala*, and *Stemphylium* were exclusively found in this group. Although no genus was specifically associated with either UC or CD, nor with disease activity, *Hypopichia* and *Metschnikowia* were identified in all IBD groups but were absent in controls. Additionally, *Aureobasidium* and *Wickerhamomyces* were present in all IBD cohorts except in active UC (Figure 7.6).

To further investigate fungal alterations in IBD, we compared the relative abundance of the top 15 genera between IBD groups and controls. In active UC, *Cladosporium* was enriched, while *Rhodotorula* was reduced compared to controls. In quiescent UC, *Cladosporium* and *Malassezia* showed a decrease relative to controls. Lastly, both active and quiescent CD biopsies exhibited a reduction in *Malassezia* and *Saccharomyces* compared to controls.

A summary of all the alterations in fungi genera in biopsies described in the different disease groups are represented in Table 7.2.

Table 7.2. Fungal alterations in IBD observed in IBD groups with respect to the controls.

Sample	Group	Changes in fungal genera (increase/decrease)

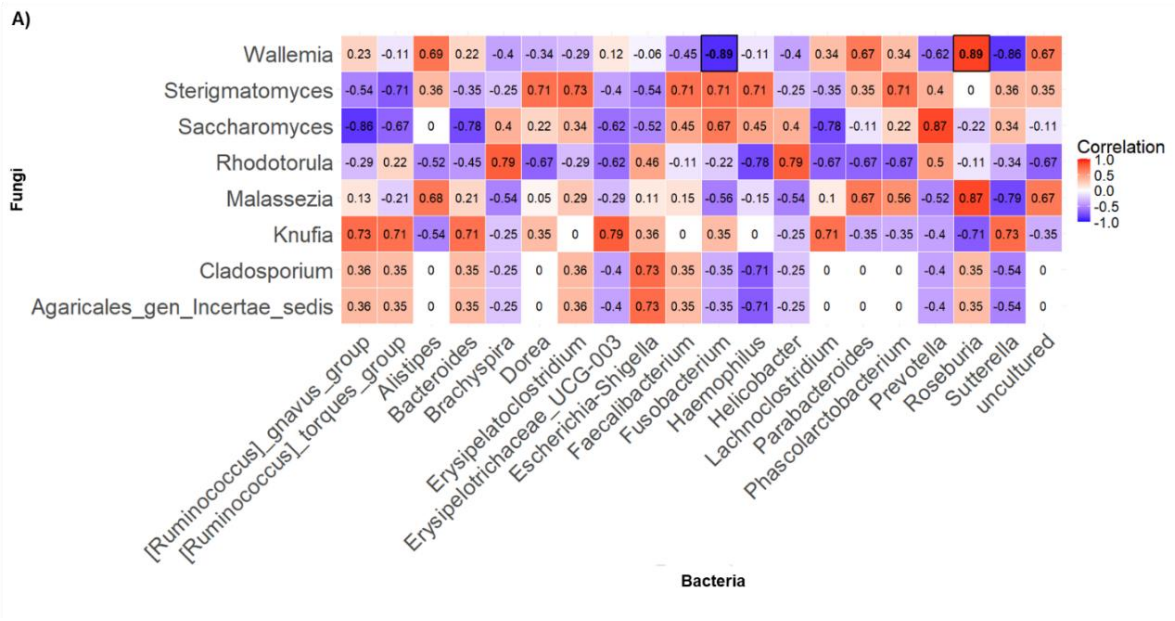
Biopsies	**Active CD**	↑ *Cercospora, Lasiobolidium, Polysporales*	↑ *Metschnikowia, Wickerhamomyces* ↓*Malassezia, Saccharomyces*
	Quiescent CD	↑ *Cystofilobasisium, Phaeosphaeriaceae, Shroeteria and Sympodiomycopsis*	
	Active UC	↓*Rhodotorula,* ↑ *Cladosporium, Malassezia, Debaryomyces, Filobasidium, Naganishia, Spizellomycetales*	↑*Pleosporales*
	Quiescent UC	↓*Cladosporium, Malassezia,* ↑ *Metschnikowia, Malassezia, Alternaria, Exophiala, Stemphylium*	

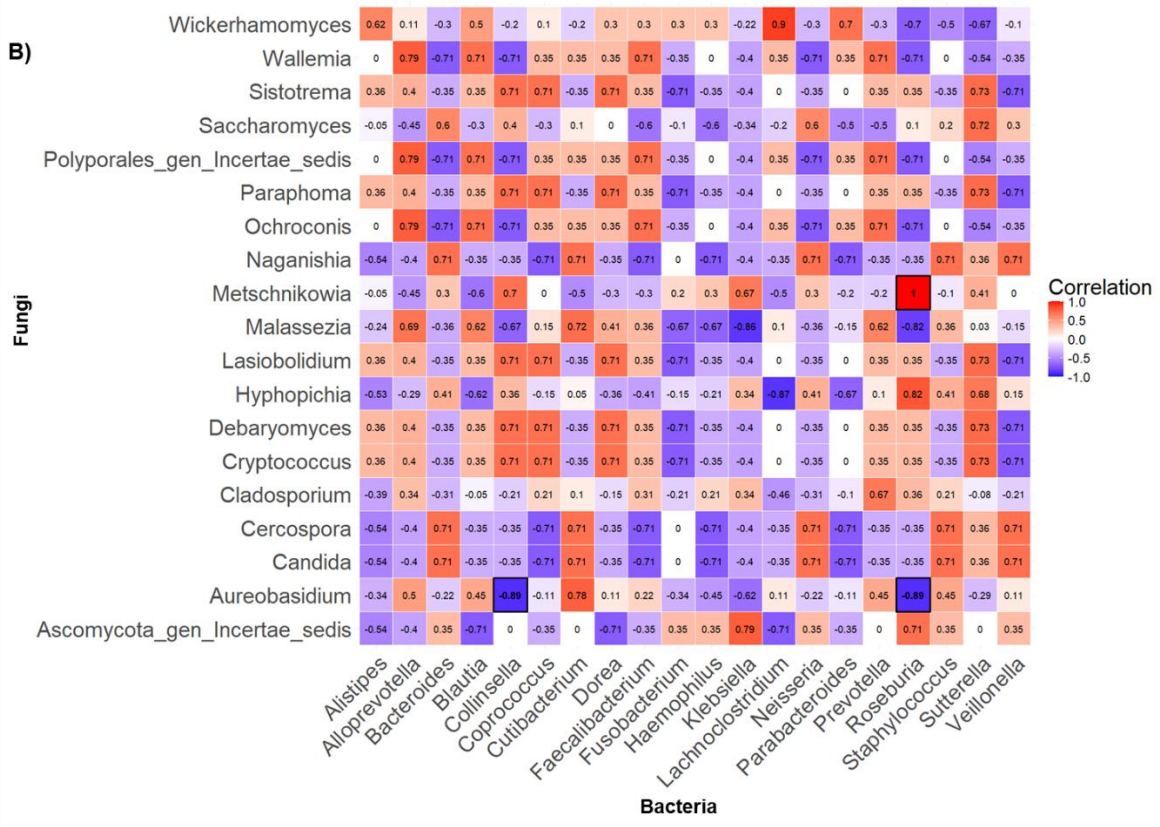
7.3.1.5 Viral composition

Similar to the mycobiome, the virome remains largely unexplored. In this study we aim to characterize the viral composition in intestinal biopsies across all cohorts. However, the low proportion of viral DNA compared to the host DNA (human) resulted in an insufficient number of reads, limiting a comprehensive analysis. As a result, viral sequences were underrepresented, limiting further analysis.

7.3.2 Microbiota correlation in intestinal biopsies

Microbial interactions within the gut ecosystem were explored through bacterial-fungal correlation analysis. Spearman's rank correlation coefficient was calculated to analyse associations between controls, active UC, quiescent UC, active CD and quiescent CD (Figure 7.7). Significant correlations and their principal characteristics in biopsies are summarized in Table 7.3. For a better comprehension of the role of the most relevant bacterial and fungal genera, Table 7.4 and Table 7.5 compile their main features and potential effects in health.


A)
Fungi
Wallemia
Sterigmatomyces
Saccharomyces
Rhodotorula
Malassezia
Knufia
Cladosporium
Agaricales_gen_Incertae_sedis
[Ruminococcus]_gnavus_group
[Ruminococcus]_torques_group
Alistipes
Bacteroides
Brachyspira
Dorea
Erysipelatoclostridium
Erysipelotrichaceae_UCG-003
Escherichia-Shigella
Faecalibacterium
Fusobacterium
Haemophilus
Helicobacter
Lachnoclostridium
Parabacteroides
Phascolarctobacterium
Prevotella
Roseburia
Sutterella
uncultured
Bacteria
Correlation
1.0
0.5
0.0
-0.5
-1.0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Fungi
Wickerhamomyces
Wallemia
Sistotrema
Saccharomyces
Polyporales_gen_Incertae_sedis
Paraphoma
Ochroconis
Naganishia
Metschnikowia
Malassezia
Lasiobolidium
Hyphopichia
Debaryomyces
Cryptococcus
Cladosporium
Cercospora
Candida
Aureobasidium
Ascomycota_gen_Incertae_sedis
Alistipes
Alloprevotella
Bacteroides
Blautia
Collinsella
Coprococcus
Cutibacterium
Dorea
Faecalibacterium
Fusobacterium
Haemophilus
Klebsiella
Lachnoclostridium
Neisseria
Parabacteroides
Prevotella
Roseburia
Staphylococcus
Sutterella
Veillonella
Bacteria
Correlation
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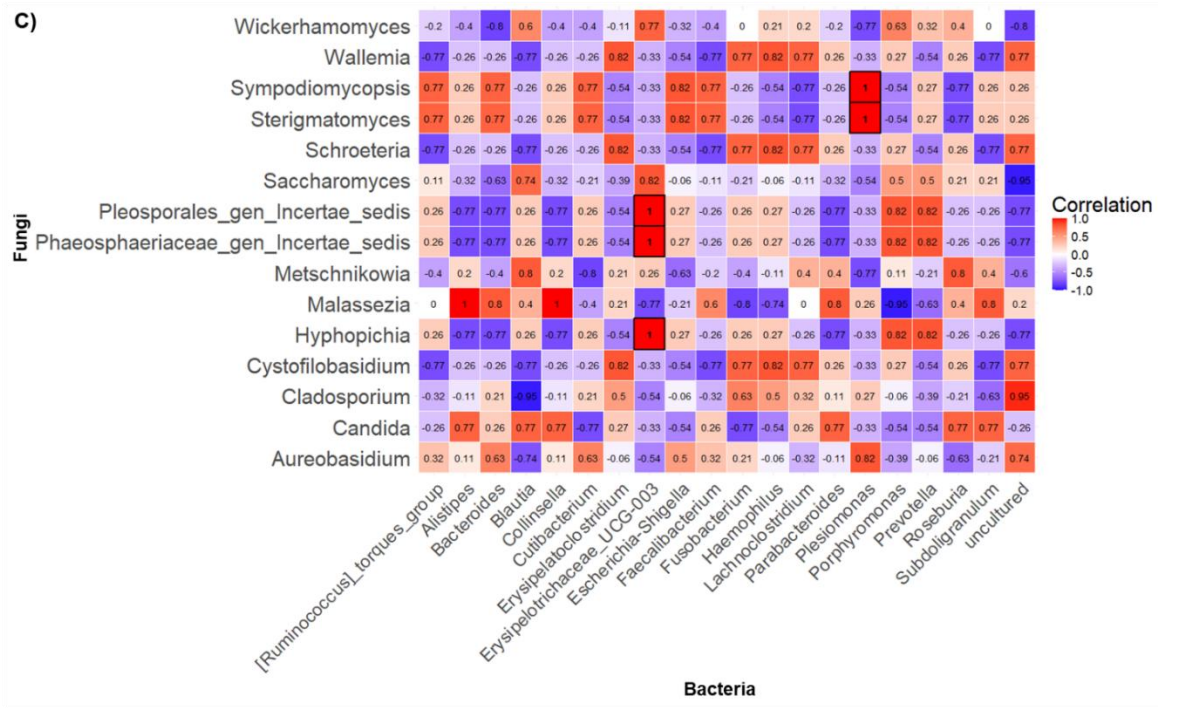
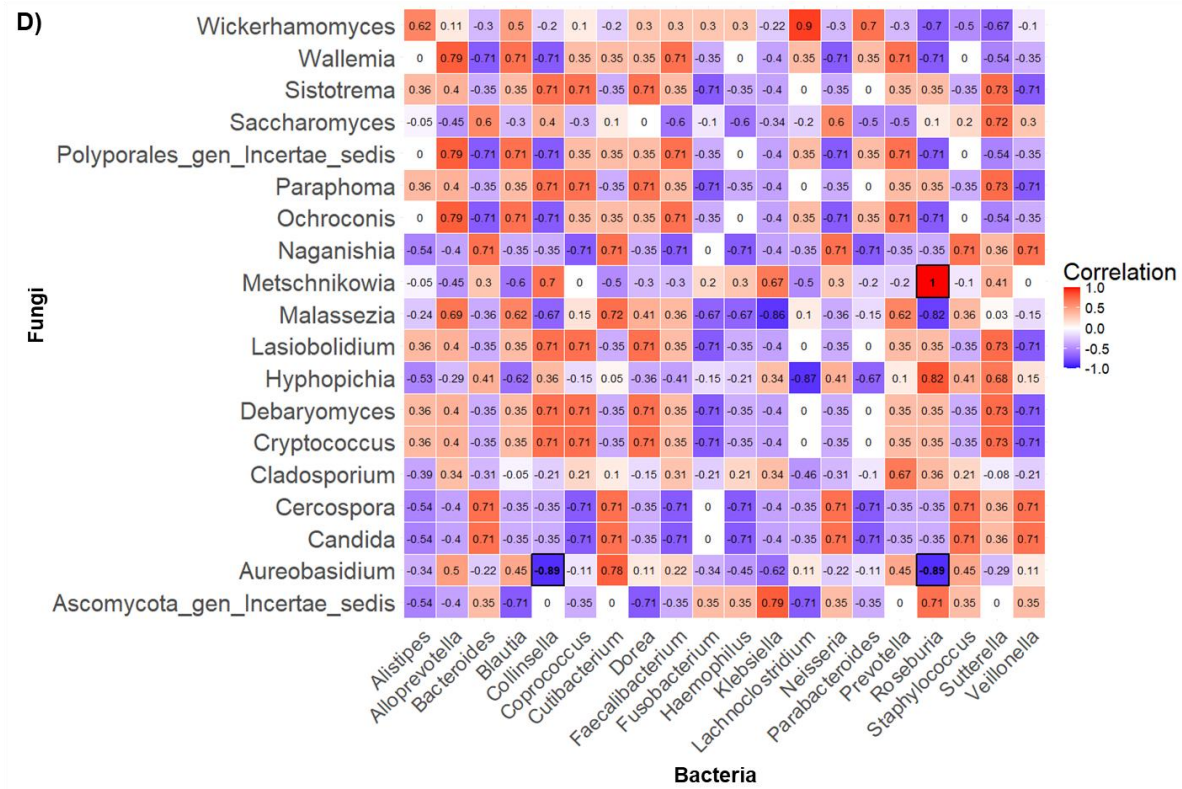

C)
Wickerhamomyces
Wallemia
Sympodiomycopsis
Sterigmatomyces
Schroeteria
Saccharomyces
Pleosporales_gen_Incertae_sedis
Phaeosphaeriaceae_gen_Incertae_sedis
Metschnikowia
Malassezia
Hyphopichia
Cystofilobasidium
Cladosporium
Candida
Aureobasidium
Fungi
[Ruminococcus]_torques_group
Alistipes
Bacteroides
Blautia
Collinsella
Cutibacterium
Erysipelatoclostridium
Erysipelotrichaceae_UCG-003
Escherichia-Shigella
Faecalibacterium
Fusobacterium
Haemophilus
Lachnoclostridium
Parabacteroides
Plesiomonas
Porphyromonas
Prevotella
Roseburia
Subdoligranulum
uncultured
Bacteria
Correlation
1.0
0.5
0.0
-0.5
-1.0


D)
Wickerhamomyces
Wallemia
Sistotrema
Saccharomyces
Polyporales_gen_Incertae_sedis
Paraphoma
Ochroconis
Naganishia
Metschnikowia
Malassezia
Lasiobolidium
Hyphopichia
Debaryomyces
Cryptococcus
Cladosporium
Cercospora
Candida
Aureobasidium
Ascomycota_gen_Incertae_sedis
Fungi
Alistipes
Alloprevotella
Bacteroides
Blautia
Collinsella
Coprococcus
Cutibacterium
Dorea
Faecalibacterium
Fusobacterium
Haemophilus
Klebsiella
Lachnoclostridium
Neisseria
Parabacteroides
Prevotella
Roseburia
Staphylococcus
Sutterella
Veillonella
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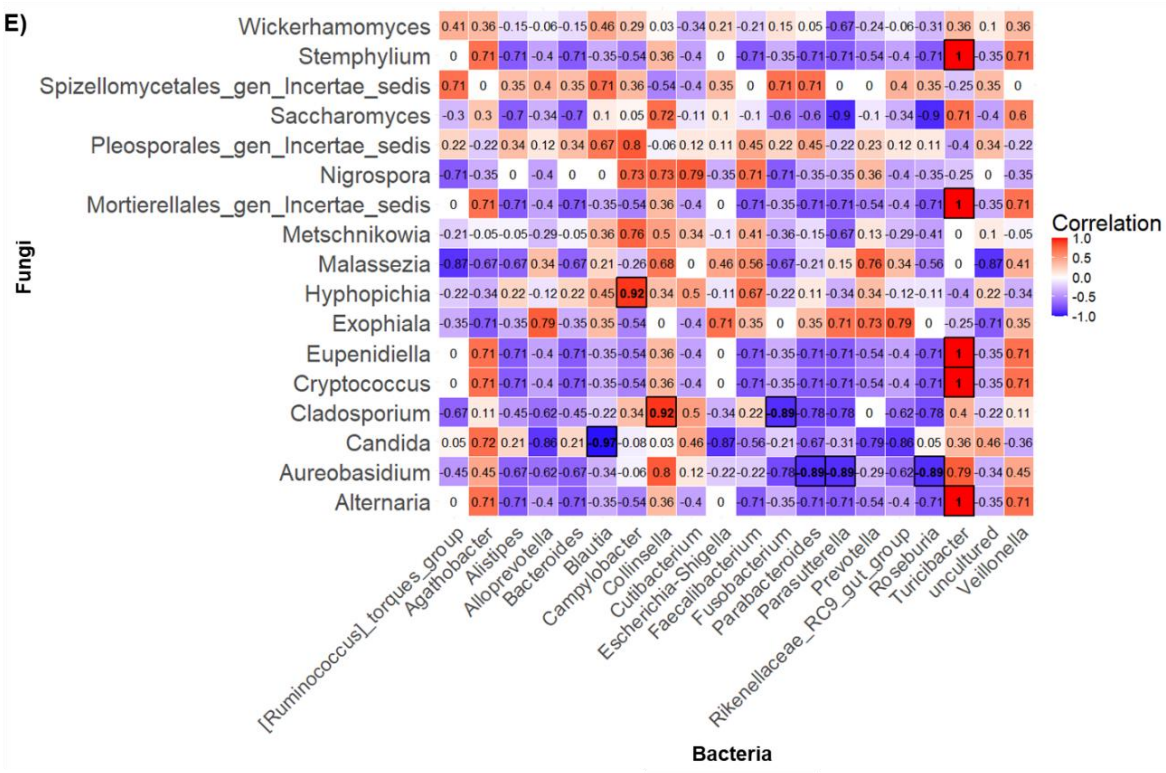

Figure 7.7. Correlations between bacteria and fungi genus in biopsies.
Spearman's rank correlation coefficient was calculated to analyse the correlations between bacteria and fungi in biopsies. Genus which a value of 0 in their relative frequency for all the patients were deleted. The hypothesis of no correlation was checked based on the asymptotic t-student to identify significant correlations. Significant correlations (p-valor < 0,05) are highlighted in bold. ***A)*** *Controls,* ***B)*** *Active Crohn,* ***C)*** *Quiescent Crohn,* ***D)*** *Active colitis,* ***E)*** *Quiescent colitis.*

Table 7.3. Significant correlations between bacteria and fungi genus (p-value < 0,05).

Group	Fungi	Bacteria	R
Control	*Wallemia*	*Fusobacterium*	-0,89
	Wallemia	*Roseburia*	0,89
Active UC	*Hyphopichia*	*Prevotellaceae*	1
	Filobasidium	*Prevotellaceae*	1
	Cladosporium	*Sutterella*	0,89
Quiescent UC	*Stemphylum*	*Turicibacter*	1
	Mortierellales	*Turicibacter*	1
	Eupenidiella	*Turicibacter*	1
	Cryptococcus	*Turicibacter*	1
	Alternaria	*Turicibacter*	1
	Hyphopichia	*Campylobacter*	0,92
	Cladosporium	*Collinsella*	0,92
	Cladosporium	*Fusobacterium*	-0,89
	Candida	*Blautia*	-0,97
	Aureobasidium	*Parabacteroides*	-0,89
	Aureobasidium	*Parasutterella*	-0,89
	Aureobasidium	*Roseburia*	-0,89
Active CD	*Metschnikowia*	*Roseburia*	1
	Aureobasidium	*Collinsella*	-0,89
	Aureobasidium	*Roseburia*	-0,89
Quiescent CD	*Sympodiomycopsis*	*Plesiomonas*	1
	Sterigmatomyces	*Plesiomonas*	1
	Pleosporales	*Erysipelotrichaceae*	1
	Phaeosphaeriaceae	*Erysipelotrichaceae*	1
	Hyphopichia	*Erysipelotrichaceae*	1

Table 7.4. Principal characteristics of most relevant bacteria genus.

Bacterial genus	Principal characteristics	Health effects	References
Agathobacter	✓Produce butyrate, a short-chain fatty acid which contributes to energy homeostasis, colonic motility, immunomodulation and suppression of gut inflammation ✓Involved in the fermentation of carbohydrates ✓Some strains can utilize a range of dietary and host-derived carbohydrates	✓Considered part of the core human gut microbiome ✓ Increased abundance associated with barley consumption, which may have positive effects on glucose tolerance ✓Less prominent in patients with ulcerative colitis compared to healthy individuals	(19,20)

Anaerostipes	✓Butyrate producer ✓Capable of fermenting carbohydrates ✓Some strains can utilize acetate and lactate to produce butyrate	✓Considered part of the core human gut microbiome ✓Some species may be involved in metabolizing the anticancer drug 5-fluorouracil	(21,22)
Bacteroides	✓Play crucial roles in breaking down complex polysaccharides ✓Capable of degrading and utilizing glycans, including mucin-type O-glycans ✓Produce short-chain fatty acids (SCFA) as fermentation end products	✓Maintain a complex and generally beneficial relationship with the host when in the gut ✓Can become opportunistic pathogens if they escape the gut environment ✓Associated with bacteraemia and abscess formation in various body sites (*B. fragilis)*	(23,24)
Bifidobacterium	✓Ferment carbohydrates, producing lactic acid and acetic acid as primary end products ✓Can break down complex carbohydrates, including oligosaccharides (prebiotics) ✓Some species can metabolize host-derived glycans, including mucin	✓Help maintain gut homeostasis ✓May provide protection against pathogens through competitive exclusion ✓Involved in modulating the immune system ✓May help in mineral absorption and protect against intestinal permeability	(25,26)
Blautia	✓Ferment carbohydrates, producing SCFAs ✓Can utilize a wide range of carbohydrates, including indigestible ones ✓Some species can use CO, H_2/CO_2, and carbohydrates as energy sources ✓Produce acetic acid, succinic acid, lactic acid, and ethanol as fermentation end products	✓Associated with both positive and negative health outcomes ✓May play a role in alleviating inflammatory and metabolic diseases ✓Shows antibacterial activity against specific microorganisms ✓Some species produce health-promoting compounds like SCFAs and antimicrobial peptides	(27,28)

Clostridium	✓Ferment carbohydrates and proteins to produce SCFAs like butyrate ✓Produce beneficial metabolites like indole propionic acid ✓Metabolize bile acids	✓Can alter differentiation of T helper 17 cells and regulatory T cells ✓ Many Clostridium species have beneficial effects: help maintain intestinal homeostasis, Strengthen the intestinal barrier and have shown to alleviate colitis and allergic diarrhea ✓ Some species can be pathogenic, for example C. difficile can cause severe diarrhea and colitis	(7,29,30)
Collinsella	✓ Metabolize bile acids to oxo-bile acid intermediates	✓ Promotion of inflammation by altering neutrophil chemotaxis and producing an increase in NF-kB ✓ *Collinsella* abundance was found to be 12-fold higher in patients with non-alcoholic steatohepatitis (NASH) compared to controls	(31)
Eubacterium hallii	✓ Produces butyrate from glucose, acetate, and lactate ✓ Capable of utilizing glycerol to produce 3-hydroxypropionaldehyde (3-HPA, reuterin) ✓ Converts 1,2-propanediol to propionate, propanal, and propanol ✓ Produces cobalamin (vitamin B12)	✓ Increases faecal butyrate concentrations ✓ Affects bile acid metabolism, potentially impacting glucose and energy homeostasis	(32,33)
Faecalibacterium	✓ Major butyrate producer in the gut ✓ Acetate consumer (acetate cross-feeding) ✓ Ferments indigestible fiber	✓ Considered a biomarker for a healthy gastrointestinal tract ✓ Decreased abundance linked to inflammatory bowel diseases (IBD) and colorectal cancer ✓ Possesses anti-inflammatory properties ✓ May act as a keystone taxon in stabilizing the gut microbiota	(34,35)

Fusobacterium	✓ Produce butyric acid as a major end product of metabolism ✓ Unable to ferment carbohydrates	✓ Part of the normal flora in the human gut mucosa, it is found particularly in the colon ✓ Implicated in colorectal cancer development ✓ Linked to inflammatory bowel diseases ✓ Can induce secretion of specific IgA antibodies	(36–38)
Lactobacillus	✓ Metabolize carbohydrates to produce lactic acid ✓ Some species can ferment indigestible fibers ✓ Aid in digestion of certain dietary substrates, including lactose	✓ Strengthen intestinal barrier function ✓ Increase mucus production ✓ Stimulate release of anti-microbial peptides ✓ Enhance production of secretory immunoglobulin A (sIgA) ✓ Increase tight junction integrity of intestinal epithelial cells ✓ Provide competitive resistance against pathogens	(39,40)
Parabacteroides	✓ Produce acetic and succinic acids as major degradation products of sugars ✓ Capable of carbohydrate metabolism ✓ Secrete short-chain fatty acids	✓ Associated with metabolic syndrome, inflammatory bowel disease, and obesity ✓ Some species (*P. distasonis and P. goldsteinii*) show potential as next-generation probiotics due to protective effects on inflammation and obesity in mice	(41,42)
Prevotella	✓ Associated with plant-rich diets high in complex carbohydrates ✓ Capable of metabolizing various plant polysaccharides	✓ Associated with both beneficial and potentially detrimental effects ✓ Linked to improved glucose metabolism and reduced visceral fat ✓ Also associated with chronic inflammatory conditions, insulin resistance, and hypertension ✓ Found in inflamed tissue in UC patients	(43–45)

Roseburia	✓ Produce SCFAs, particularly butyrate ✓ Ferment complex polysaccharides ✓ Prefer an acidic intestinal environment	✓ Part of the normal gut microbiota, primarily inhabit the human colon ✓ Produced butyrate serves as an energy source for colonocytes ✓ Helps maintain gut barrier function	(46,47)
Ruminococcus gnavus	✓ Capable of utilizing both dietary carbohydrates and host-derived sugars ✓ Some strains have evolved to preferentially use sugars found in the gut lining	✓ Associated with both health and disease states ✓ Increased abundance linked to various intestinal disorders (IBD, IBS, colon cancer) ✓ Also associated with extra-intestinal conditions (skin allergies, cardiovascular diseases, liver diseases, brain disorders)	(48,49)
Ruminococcus torques	✓ Capable of degrading mucin glycoproteins and O-linked glycans ✓ Utilizes both mucin glycoproteins and released oligosaccharides from gastric and colonic mucins ✓ Possesses strong fucosidase, sialidase, and β1,4-galactosidase activities ✓ Lacks detectable sulfatase activity and has weak β1,3-galactosidase activity ✓ Secretes a variety of enzymes which are involved in the degradation of mucin and mucin-derived glycans	✓ Associated with inflammatory bowel diseases (IBDs) in multiple studies ✓ Its mucin-degrading ability may contribute to defects in mucus protection ✓ Potential target for preventing or treating IBD due to its keystone role in mucin degradation	(50)
Shigella	✓ Generally, cannot ferment lactose ✓ Produce organic acids from carbohydrate or peptone metabolism	✓ Highly pathogenic, causing shigellosis (bacillary dysentery) ✓ Invade the epithelial lining of the colon, causing severe inflammation and cell death ✓ Trigger release of pro-inflammatory cytokines (IL-1β, IL-18) ✓ Suppress innate immune responses ✓ Interfere with adaptive immune responses, leading to partial susceptibility to re-infection	(51,52)

Turicibacter	✓ Produce short-chain fatty acids, primarily lactate, with smaller amounts of acetate and butyrate	✓ Primarily found in the gut ✓ Different *Turicibacter* strains exhibit varying abilities to modify bile acids ✓ Possess bile salt hydrolases (BSHs), so they may modulate serum bile acid profile ✓ Can influence host lipid and cholesterol metabolism	(53–55)

Table 7.5. Principal characteristics of most relevant fungi genus.

Fungi genus	Principal characteristics	Health effects	References
Aureobasidium	✓ They can produce a wide variety of compounds, including enzymes, polysaccharides, and biosurfactants ✓ Some species can ferment to produce β-polymalic acid, laccase, liamocins, and pullulan polysaccharides	✓ Its presence in the gut would likely be transient or incidental rather than as an established colonize	(56,57)
Candida	✓ *Candida* species can ferment various sugars, with different species having distinct fermentation profiles ✓ They can adapt to different nutrient environments within the gut	✓ It is part of the normal flora of the gastrointestinal tract in many healthy individuals ✓ *Candida* species interact with the host immune system and can modulate immune responses ✓ Overgrowth of *Candida* in the intestine has been linked to various gastrointestinal disorders, including inflammatory bowel disease and irritable bowel syndrome ✓ In mice, can induce protective immune responses against invasive candidiasis, mediated by elevated systemic anti-C. albicans Th17 cells and IL-17 responsive neutrophils	(58,59)

Cladosporium	✓ Can produce cladosporide A, an antifungal agent against the human pathogenic filamentous fungus Aspergillus fumigatus	✓ It is a common opportunistic fungus with the ability to colonize the gastrointestinal tract ✓ Over half of the natural products isolated from *Cladosporium* have been found to have various biological activities, including cytotoxic, antibacterial, antiviral, antifungal and enzyme-inhibitory activities	(60,61)
Exophiala	✓ *Exophiala* can produce three different types of melanin ✓ *Exophiala* species are polyextremotolerant, able to survive in harsh environments	✓ While not typically associated with the gut, *Exophiala* has been found to colonize the human intestine and respiratory tract ✓ In the gut of UC patients there are less amount of *Exophiala* compared to healthy controls	(62,63)
Filobasidium	✓ *Filobasidium* species can ferment various sugars ✓ They are known to assimilate a wide range of carbon compounds ✓ Some species produce extracellular enzymes that could potentially aid in nutrient acquisition	✓ The enriched presence of *Filobasidium* spp. in donor faeces is associated with the positive response to Faecal microbiota transplantation (FMT) for patients with UC ✓ *Filobasidium* species were found near non-inflamed tissue in biopsies from Crohn patients	(64,65)
Malassezia	✓ *Malassezia* species are lipophilic, meaning they require lipids for growth	✓ *Malassezia* is part of the human mycobiome ✓ They can modulate immune responses, particularly the innate immune system ✓ It can interact with pattern-recognition receptors (PRRs) like Toll-like receptors (TLRs), leading to the release of cytokines ✓ Higher prevalence of Malassezia in the intestines of patients with Crohn's disease, where it might trigger immune responses contributing to inflammation	(66,67)

Rhodotorula	✓ They produce the enzyme urease and do not ferment carbohydrates ✓ *Rhodotorula* species produce carotenoids	✓ *Rhodotorula* species are commonly found in the human gastrointestinal tract ✓ They may have a probiotic effect by regulating the multiplication of pathogenic bacteria and neutralizing or destroying their toxins ✓ Colonized humans may benefit from nutrients produced by *Rhodotorula*, including proteins, lipids, folate, and carotenoids ✓ Some strains of *Rhodotorula* are being studied for their potential beneficial effects on immune function and gut microbiota	(68,69)
Saccharomyces	✓ *S. cerevisiae* can ferment various sugars ✓ It produces enzymes like urease and does not ferment carbohydrates ✓ Produce antimicrobial peptides, modulate the immune system, and have trophic effects	✓ *Saccharomyces* species can influence the composition of the gut microbiota, potentially increasing beneficial bacteria and reducing harmful ones ✓ *S. boulardii* is widely used as a probiotic for treating gastrointestinal disorders, particularly diarrhea ✓ It can improve gut barrier function ✓ *Saccharomyces* is mostly associated with an anti-inflammatory effect on dendritic cells, as well as suppression of the exacerbated activation of the NLRP3 inflammasome both in patients and in murine models of IBD	(70–72)
Wallemia	✓ *Wallemia* species can produce toxins even under saline conditions ✓ They are considered filamentous food-borne pathogenic fungi	✓ While not primarily considered intestinal fungi, *Wallemia* species can be ingested through contaminated food. ✓ *W. sebi, W. mellicola, and W. muriae* have been reported to be related to human health problems.	(73)

7.3.3 Microbial diversity in faeces

7.3.3.1 Bacterial diversity in faeces

Bacterial communities in the human microbiota have typically been studied either in faecal samples or intestinal biopsies separately. To provide a more comprehensive view of the human bacteriome, bacterial composition of the faeces of the same patients whose mucosal samples had been previously examined was analysed.

First, to assess bacterial diversity in intestinal biopsies, alpha diversity was analysed using both Shannon and Simpson indices (Figure 7.8). Kruskal Wallis test was also performed but no significative differences among groups were observed (Shannon or Simpson index).

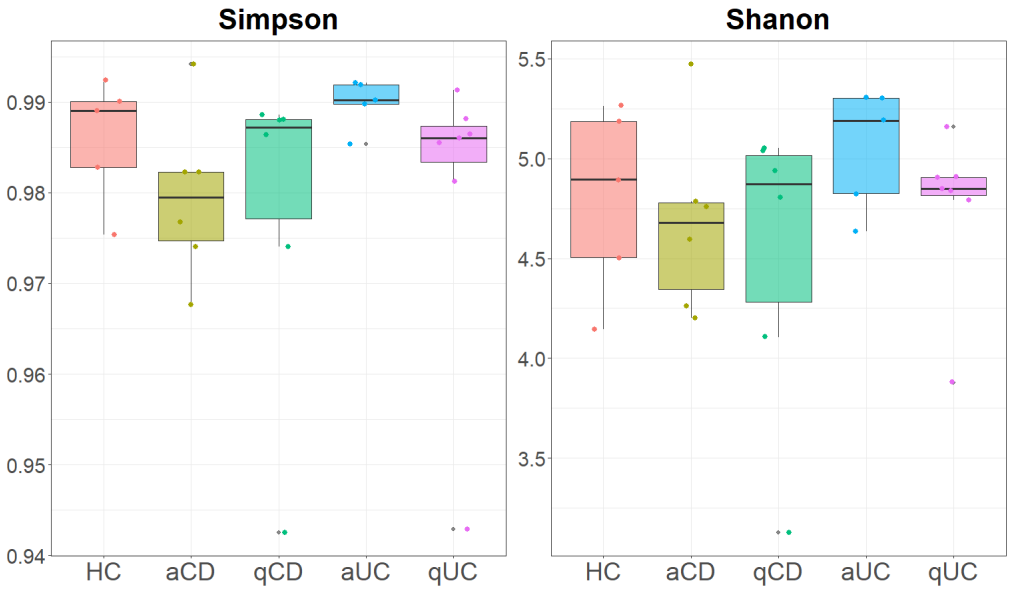

Figure 7.8. Bacterial alpha diversity in stool samples.
Shannon and Simpson indices of alpha diversity of the bacteria were calculated in stool samples from the different studied groups: healthy controls (HC), active Crohn's disease (aCD), quiescent Crohn's disease (qCD), active colitis (aUC) and quiescent colitis (qUC). Kruskal Wallis test was then performed. P-value < 0.05 was considered significant.

Alpha diversity was very similar between groups (as indicated similar values in both Shannon and Simpson indices), however in controls, Shannon and Simpson values showed greater microbial diversity with a balanced microbiome and no dominance by specific species while a reduction in microbial diversity was observed during the active phases of CD and UC. Finally, in both quiescent CD and UC samples a little recovery of the dysbiosis with no dominance by any specie was observed.

Beta diversity was evaluated using Bray Curtis, Jaccard and Unifrac (weighted and unweighted) distances metrics. No significant differences were observed between groups (Figure 7.9).

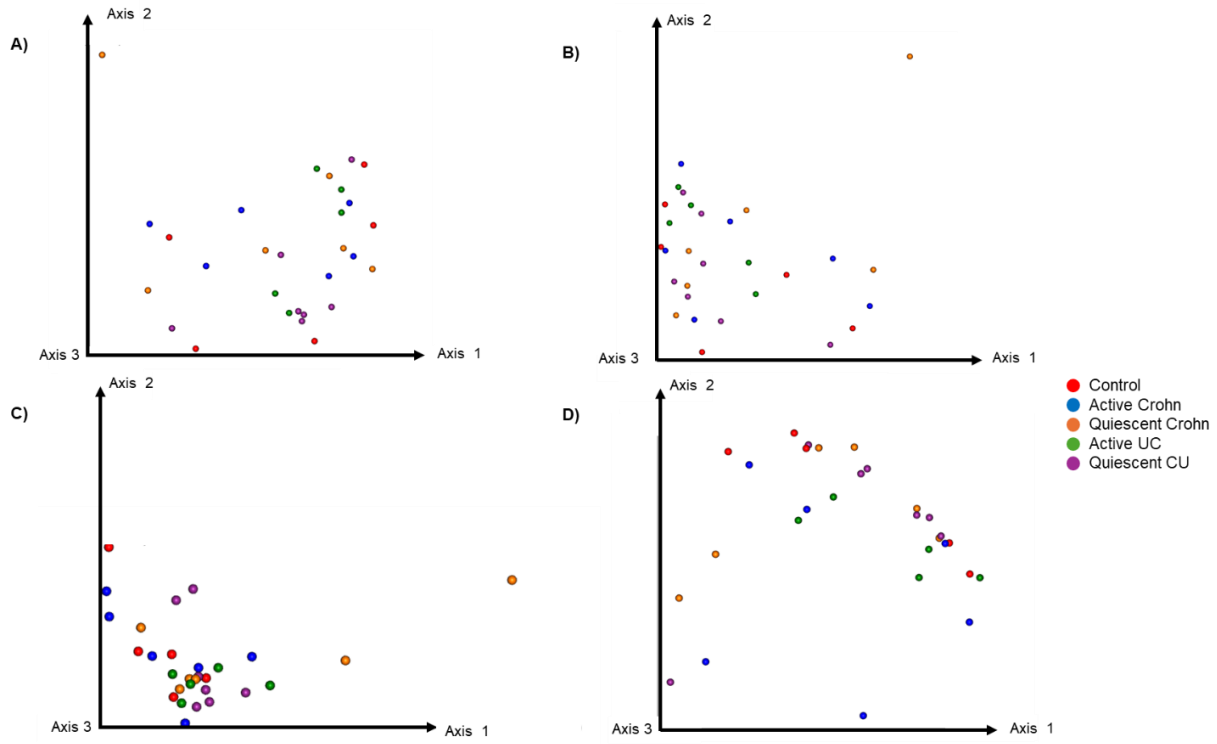

Figure 7.9. Bacterial beta diversity in stool samples.
*PCoA showing beta diversity was calculated using different distances: **A)** Bray Curtis, **B)** Jaccard, **C)** Unweighted Unifrac, **D)** Weighted Unifrac.*

7.3.3.2 Bacterial composition in faeces

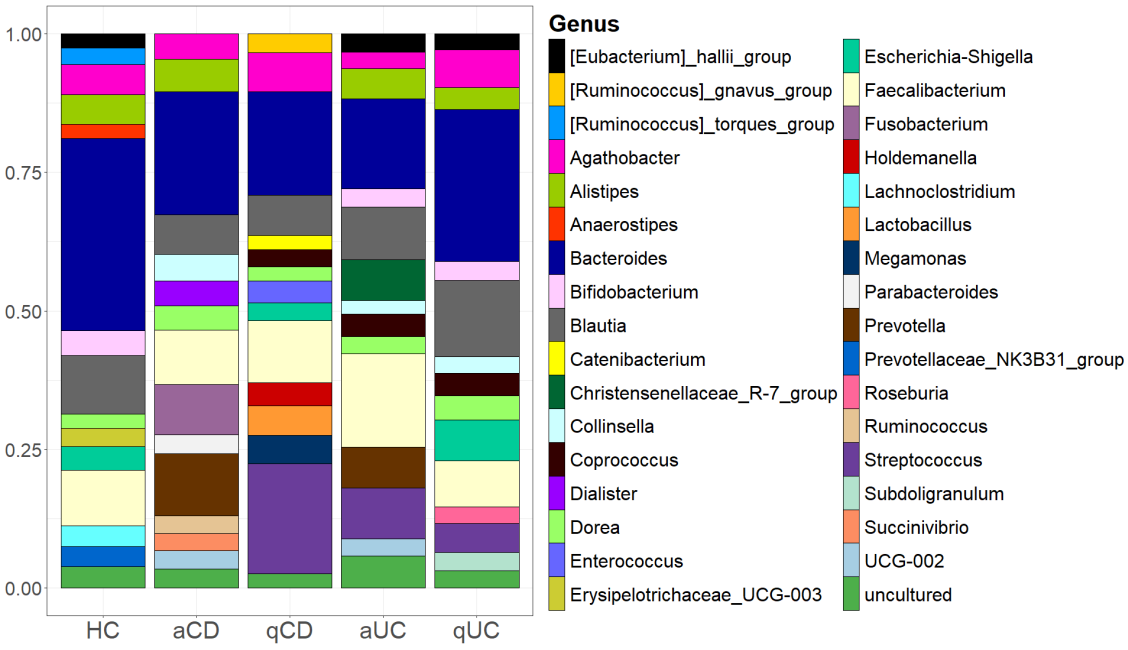

Figure 7.10. Top 15 genus of bacteria in stool samples.
The 15 most abundant bacteria genera found in stool samples were identified for each group: healthy controls (HC), active Crohn's disease (aCD), quiescent Crohn's disease (qCD), active colitis (aUC) and quiescent colitis (qUC). Relative frequencies of each genus were calculated and those which relative frequencies were not assigned to any genus (N/A) were discarded. Values of these 15 genera were standardized to 0-1 to calculate the percentage that is represented.

Our results showed that *Bacteroides*, *Blautia*, and *Faecalibacterium* were the most prevalent genera, with *Streptococcus* also being highly represented in quiescent CD stool samples. Distinct bacterial signatures were observed across groups. In controls, five genera were exclusively identified: *Ruminococcus torques*, *Anaerostipes*, *Erysipelotrichaceae*, *Lachnoclostridium*, and *Prevotellaceae*. In active CD, *Dialister*, *Fusobacterium*, *Parabacteroides*, *Ruminococcus*, and *Succinivibrio* were uniquely present, while quiescent CD samples exhibited five specific genera: *Ruminococcus gnavus*, *Catenibacterium*, *Enterococcus*, *Holdemanella*, *Lactobacillus*, and *Megamonas*. UC stools had fewer exclusive genera, with *Christensenellaceae* found only in active UC and *Roseburia* and *Subdoligranulum* restricted to quiescent UC. Notably, *Prevotella* was detected solely in active disease states, both in CD and UC.

To further explore bacterial alterations in IBD, differences in the abundance of representative genera between IBD groups and controls were analysed. Stool samples showed greater similarity among cohorts and fewer variations in genus abundance. The most striking change was a decrease in *Bacteroides* across all IBD groups compared to controls. Additionally, *Coprococcus* and *Streptococcus* were present in all IBD groups except active CD, while *Collinsella* was detected in all IBD cohorts except quiescent CD.

A summary of all the alterations in bacterial genera, both in faeces and biopsies described in the different disease groups are found in Table 7.6.

Table 7.6. Bacterial dysbiosis in IBD groups with respect to the controls both in biopsies and stool.

Sample	Group	Changes in bacterial genera (increase/decrease)

stool	**Active CD**	↑ Collinsella, *Dialister, Fusobacterium, Parabacteroides, Ruminococcus, Succinivibrio, Prevotella*	↓ *Bacteroides, Anaerostipes, Ruminococcus torques, Erysipelotrchaceae, Lachnoclostridium, Prevotellaceae*
	Quiescent CD	↑ *Ruminococcus gnavus, Catenibacterium, Coprococcus, Enterococcus, Holdemanella, Lactobacillus, Megamonas, Streptococcus*	
biopsies	**Active CD**	↑ *Alloprevotella, Neisseria, Staphylococcus, Cutibacterium, Prevotella, Veillonella,* ↓ *Escherichia-Shigella*	↑ *Fusobacterium, Faecalibacterium, Alistipes* ↓ *Brachyspira, Erysipelotrichaceae, Ruminococcus gnavus*
	Quiescent CD	↑*Alistipes, Collinsella, Plesiomonas, Porphyromonas, Prevotella, Subdoligranulum*	
Stool	**Active UC**	↑ *Christensenellaceae, Coprococcus, Prevotella, Faecalebacterium, Collinsella, Streptococcus*	↓ *Bacteroides, Anaerostipes, Ruminococcus torques, Erysipelotrchaceae, Lachnoclostridium, Prevotellaceae*
	Quiescent UC	↑ *Collinsella, Coprococcus, Escherichia-Shigella, Roseburia, Subdoligranulum, Streptococcus*	
Biopsies	**Active UC**	↑ *Bacteroides, Faecalibacterium, Lachnoclostridium, Roseburia, Sutterella, Clostridium innocuum, Dorea, Anaerostipes*	↑ *Faecalibacterium, Alistipes, Turicibacter* ↓ *Brachyspira, Erysipelotrichaceae, Ruminococcus gnavus*
	Quiescent UC	↑ *Agathobacter, Alloprevotella, Blautia, Campylobacter, Faecalibacterium, Parabacteroides, Parasutterella, Veillonella* ↓ *Escherichia-Shigella*	

7.3.4 Microbiota comparison between different groups

To assess differences between groups (controls, active and quiescent UC, active and quiescent CD) PERMANOVA (Permutational Multivariate Analysis of Variance) was

performed in a pairwise manner. PERMANOVA was conducted comparing two groups each time using Bray Curtis distances and was repeated for each analysis (16S faeces, 16S biopsies and ITS2 biopsies). Results are summarized in Table 7.7. There were only significative differences in fungi diversity between control vs active CD and active UC vs active CD, pseudo-F value in both comparisons is high (3,6402 and 3,836 respectively) indicating that the variability between groups was significantly greater than the variability within groups.

Table 7.7. PERMANOVA tests among all groups.

PERMANOVA	**Fungi Biopsies**		**Bacteria Biopsies**		**Bacteria Stool**	
Groups	Pseudo-F	P-Value	Pseudo-F	P-Value	Pseudo-F	P-Value
Control-Active Colitis	0,2894	0,971	0,8094	0,771	1,106	0,327
Control-Quiescent Colitis	1,2474	0,252	1,013	0,42	0,6201	0,877
Control-Active Crohn	3,6402	**0,005***	1,1926	0,221	0,8029	0,807
Control-Quiescent Crohn	1,5191	0,101	0,8541	0,669	0,7717	0,78
Active Colitis-Quiescent Colitis	1,4056	0,198	0,8472	0,774	1,0633	0,36
Active Colitis-Active Crohn	3,836	**0,009***	1,3829	0,15	0,8897	0,633
Active Colitis-Quiescent Crohn	1,719	0,16	1,1298	0,32	0,584	0,986
Quiescent Colitis-Active Crohn	1,4249	0,198	1,0821	0,31	1,2079	0,172
Quiescent Colitis-Quiescent Crohn	0,7634	0,701	0,7512	0,859	0,8013	0,729
Active Crohn-Quiescent Crohn	0,7297	0,647	0,7804	0,734	0,6452	0,976

To analyse the similarities among the different groups ANOSIM (analysis of similarities) was performed in a pairwise manner. ANOSIM was done comparing two groups each time using Bray Curtis distances and was repeated for each analysis (16S faeces, 16S biopsies and ITS2 biopsies). Results are summarized in Table 7.8. There were significative differences in fungi diversity between control vs active CD and active UC vs active CD and in two cases the statistic R value were relatively close to 1 (0,49 and 0,496 respectively), which meant that differences between groups were significantly greater than differences within groups, according to PERMANOVA results. The ANOSIM test also found significative differences when comparing quiescent UC vs active CD in bacterial analysis in stool.

Table 7.8. ANOSIM tests among all groups.

ANOSIM	Fungi Biopsies		Bacteria Biopsies		Bacteria Stool	
Groups	Statistic R	P-Value	Statistic R	P-Value	Statistic R	P-Value
Control-Active Colitis	-0,164	0,902	-0,076	0,743	0,012	0,442
Control-Quiescent Colitis	0,05	0,256	0,036	0,314	-0,1336	0,853
Control-Active Crohn	0,49	**0,01***	0,124	0,153	-0,104	0,966
Control-Quiescent Crohn	0,098	0,172	-0,0625	0,657	-0,1467	0,993
Active Colitis-Quiescent Colitis	0,124	0,184	-0,02	0,589	0,0765	0,255
Active Colitis-Active Crohn	0,496	**0,012***	0,116	0,203	-0,09333	0,86
Active Colitis-Quiescent Crohn	0,08438	0,227	0,075	0,297	-0,09067	0,898
Quiescent Colitis-Active Crohn	0,076	0,169	0,008	0,449	0,1521	**0,045***
Quiescent Colitis-Quiescent Crohn	-0,05	0,671	-0,125	0,819	0,08598	0,143
Active Crohn-Quiescent Crohn	-0,0125	0,499	-0,01875	0,503	-0,1	0,996

7.3.5 Microbiota comparison between different tissues

PERMANOVA was conducted comparing faeces and biopsies in each group using Bray Curtis distances. Results are summarized in Table 7.9. The PERMANOVA test showed that faeces and biopsies have different compositions in all groups. High Pseudo-F values (1.5 or higher) in all groups indicated that there were great differences between tissues except in the case of quiescent CD.

Table 7.9. PERMANOVA tests between stool and biopsies in all groups using Bray Curtis distances from bacterial data.

PERMANOVA	Bacteria - Biopsies Vs Stool	
Group	Pseudo-F	P-Value
Control	1,7973	**0,045***
Active Colitis	2,3399	**0,016***
Quiescent Colitis	1,9253	**0,004***
Active Crohn	1,5964	**0,036***
Quiescent Crohn	1,4691	0,1

ANOSIM test was conducted between faeces and biopsies in each group using Bray Curtis distances. Results are summarized in Table 7.10. There were significative differences in controls and both active and quiescent UC groups when comparing stools to biopsies. Statistic R value was relatively close to 1 in both colitis groups (0,41 and 0,48 respectively), which meant that differences between tissues were significantly greater than differences within tissues. This R value was very close to 0 in the active CD group, indicating that the difference between tissues was small and not significant.

Table 7.10. ANOSIM tests between stool and biopsies in all groups using Bray Curtis distances from bacterial data.

ANOSIM	Bacteria - Biopsies Vs Stool	
Group	Statistic R	P-Value
Control	0,28	**0,042***
Active Colitis	0,412	**0,015***
Quiescent Colitis	0,4839	**0,002***
Active Crohn	0,088	0,189
Quiescent Crohn	0,0635	0,263

Analyzing the differences and similarities at the taxonomy level between tissues (Figure 7.3 and Figure 7.10), some genera were represented in both tissues in several groups, as *Collinsella* and *Prevotella,* both present in active status of IBD diseases. In a similar way, *Escherichia-shigella* was represented in controls and quiescent phases of IBD, both in biopsies and stools. *Bacteroides* and *Faecalebacterium* were present in all cohorts both in stool and mucosal samples. It is also interesting that *Parabacteroides* were found in mucosal samples from all cohorts except active CD, although in faeces it was found only in active CD group. *Alistipes* genus was identified in the mucosal samples from all cohorts while in faeces was only absent in quiescent CD. *Erysipelotrichaceae_UCG_003* however was present in both tissues also but only in the control groups. Parallelly, some genera were only found in one tissue as *Eubacterium_hallii* and *Bifidobacterium*, only present in faeces, or *Turicibacter* or *Veillonella*, only present in intestinal biopsies.

7.4 DISCUSSION

Our study provides novel insights into the diversity and interactions of bacterial and fungal communities in inflammatory bowel disease (IBD) at the gut location, revealing distinct microbial patterns associated with disease activity and remission. Moreover, our study analysed bacterial composition in both intestinal tissue and faecal samples from the same patients, providing a highly interesting approach to compare mucosa-associated and faecal microbiota. This paired-sample strategy enhances our understanding of microbial dynamics and explores the potential of faecal bacteria as non-invasive biomarkers for disease. A key finding was the identification in faeces of potential biomarkers, such as *Prevotella* for active disease and *Roseburia* for remission states which offers exciting opportunities for developing less invasive diagnostic tools and improving patient management.

Our results showed a decrease in SCFA producers in both CD and UC which has also been reported by other studies (74,75) and involves a reduction in the production of butyrate, propionic acid among others that promotes a tolerogenic environment in the gut (76,77). IBD involves an increase in other SCFA producing bacteria like *Turicibacter*, *Faecalebacterium*, *Alistipes* confirmed by other authors (78–80). One of their primary roles is the production of SCFAs, particularly *Faecalibacterium*, a butyrate-producer. While *Turicibacter* and *Alistipes* also produce SCFAs, they may produce different types (lactate and propionic acid, respectively) or amounts of them. Indeed, *Alistipes* has been linked to both protective and pathogenic effects as it showed protective benefits against colitis but has also been associated with colorectal cancer development (34,35,53–55,81). The depletion of SCFA producing bacteria observed in IBD patients is associated with aberrant immune responses and impaired intestinal barrier integrity (82). Analysis of the fungal diversity showed an increase in the fungi diversity of IBD patients compared with controls. This increase has been observed by other groups (83). We hypothesize that the bacterial dysbiosis in IBD facilitates fungal colonization, a process not observed in healthy individuals, as has been supported by studies showing an increase in the relative abundance of *Candida albicans* and a decrease in *Saccharomyces cerevisiae* in IBD patients compared to controls (15,84). Furthermore, increased fungal diversity in inflamed mucosa of CD patients has been correlated with disease activity and higher levels of pro-inflammatory cytokines, such as TNF-α and IFN-γ (85).

These findings suggest that IBD-related dysbiosis creates an environment that facilitates fungal colonization, although further research is necessary to fully elucidate the fungal dysbiosis associated to IBD and its impact on the pathogenesis. Regarding to that, our data suggest that *Wallemia* could play a role in balancing that presence of potentially beneficial

of pathogenic bacteria such as *Roseburia* and *Fusobacterium*. It is interesting the fact that SCFA producers like *Turicibacter*, *Roseburia* or *Erysipelotrichaceae* were detected in the same mucosal sites as potentially pathogenic fungi like *Stemphylum*, *Cladosporium* or *Aureobasidium* in remission states of the disease -both UC and CD-, indicating a mechanism of regulation of the fungi and bacteria as it has been described in other fungi (61). Some authors confirmed that SCFAs, such as acetate, propionate, and butyrate, produced by commensal intestinal bacteria, directly modulate the presence and behaviour of intestinal fungi, particularly *Candida albicans*, inhibiting its growth and its ability to form hyphae (a more invasive form) (86,87). This regulation is crucial for maintaining fungal eubiosis and preventing dysbiosis associated with inflammation or disease (61). Our results support the idea of the regulation of mycobiome and bacteriome for its own. In summary, while established microbial interactions contribute to shaping the mycobiome, the metabolic complexity of the gut microbiota likely involves additional regulatory mechanisms.

Bacterial biomarkers present a promising, less invasive alternative to traditional procedures such as colonoscopy (88,89), potentially offering a more patient-friendly approach in the future. To identify these biomarkers in our IBD patients, we assessed bacterial genera that were present in both stool and biopsy samples within the same patient groups (Figure 7.3 and Figure 7.10). In this way, *Prevotella* was identified as a potential biomarker for active status of IBD -both UC and CD-, which agrees with other studies that found pro-inflammatory characteristics of this genus (43–45); *Fusobacterium* for active CD and which was also linked with colorectal cancer and IBD (36–38) and *Roseburia,* a well-known SCFA producer related to normal microbiota (46,47), for quiescent UC. Summarizing, our results justify further investigation of these genera as potential biomarkers of IBD.

Our results highlight the critical role of bacterial–fungal balance in modulating the gut microbiota of patients with IBD. Despite limitations as the sample size and the inability to characterize the virome, we have proposed three bacteria genera that could act as biomarkers of IBD.

7.5 BIBLIOGRAPHY

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Chapter 8. Discussion

This thesis was aimed to deepen our understanding of the key factors underlying IBD, focusing primarily on the study of two main factors underlying IBD: immunome and microbiota. Regarding the immunome, I focused on APCs, as they are crucial regulators of intestinal homeostasis. In particular, cDCs play a pivotal role in shaping the type of response elicited against antigens (tolerogenic against commensals and inflammatory against pathogens, in health conditions), which led us to hypothesize that they may also be therapeutic targets of some drugs (specifically tofacitinib), used in IBD treatment (UC in this case). Furthermore, given the importance of the gut microbiota in maintaining intestinal homeostasis and its involvement in IBD pathogenesis, this thesis also aimed to characterize the faecal and intestinal mucosal-associated microbiome in human samples, with the final goal of identifying bacterial and fungal patterns and potential faecal biomarkers.

8.1 IMMUNOME STUDY

To that end, in the first instance, a protocol for isolating human intestinal LPMC from biopsies and intestinal resections has been optimized. This protocol subsequently allowed the identification and isolation of different subsets of intestinal APC, including cDC1, cDC2, $CD103^+$ cDC2, monocytes, and MΦ. This optimization is highly relevant because studies of intestinal APCs have been usually performed in mice or human cells derived from monocytes (e.g., MO-DC or MO-MΦs), which, although share some of their properties, they do not fully replicate the properties of *bona fide* intestinal DCs and MΦ. Research regarding human gut DC is limited, primarily due to methodological difficulties in studying human tissue and isolating human gut APC. For this reason, the optimization of a protocol that enables the isolation of human intestinal APC implies an advance in the study of human intestinal cDC and MΦ, and consequently, their implication in IBD, main topic in this thesis.

Focusing on the functional differences between human and mouse cDC, it becomes clear why studying human intestinal cDCs directly is essential. Although mouse gut cDC subsets are well characterized and have been key in understanding basic immunological principles, their functional responses often diverge from those of human intestinal cDC (1). For example, while murine $CD103^+$ cDCs are robust inducers of regulatory T cells via RA and TGF-β production and are capable of suppressing colitogenic responses *in vivo* (2–5), evidence for these same functions in human intestinal DCs is much more limited. Additionally, inflammatory responses show variation: murine cDCs respond strongly to TLR2 and TLR4 ligands during colitis, producing cytokines like IL-12 (6–8), whereas human intestinal DCs in IBD tissues tend to express different activation profiles, including elevated IL-6 and IL-18, with unclear upstream triggers (9,10). Moreover, human cDCs are exposed to a far more complex and variable microbiota, diet, and genetic background than lab-housed mice, which further impacts their function and plasticity (11). These differences underscore the necessity of working with primary human intestinal cDCs when the goal is to understand their precise roles in health and disease, especially in those whose cause remains unclear, as occurs in IBD.

Importantly, immunomodulatory drugs that are effective in human IBD sometimes fail to reproduce the same effects in murine models. For example, tofacitinib has been shown to ameliorate colitis severity in murine models, including dextran sulphate sodium (DSS)- and T cell transfer-induced colitis, but typically fails to induce full remission or prevent disease relapse (12). Additionally, tofacitinib have been shown to suppress pro-inflammatory cytokines such as TNF-α, IL-6, and IL-8 in colonic organoids from tissue biopsies, specifically targeting gut-resident phagocytes without disrupting basal antigen presentation (13). Another important distinction lies in the use of MO-DCs as simulators of human

intestinal APCs. Although MO-DCs are widely used due to accessibility and ease of generation, they differ transcriptionally, phenotypically, and functionally from tissue-resident *bona fide* cDCs (14). For instance, tofacitinib impairs T cell priming and reduces costimulatory molecule expression in MO-DCs derived from healthy blood donors and decrease stimulatory capacity of MO-DC (15), however in this thesis is showed that intestinal cDCs are not affected by tofacitinib neither their phenotype, nor their function (tofacitinib do not modulate their stimulatory capacity over T cells) (16), reinforcing the idea that conclusions drawn from MO-DC models may not fully replicate tissue-specific responses. In summary, experiments using primary human intestinal APCs can faithfully capture the complexity of human immune responses and allows to consider the interindividual human variability.

Having optimized a protocol that allowed the isolation and use of human intestinal APC, I explored the immunome as a key factor underlying IBD. Within the human immunome, I focused in cDC as they are key players in immune regulation determining the type of response (tolerogenic or proinflammatory) that is generated to different intestinal stimuli. Firstly, the distribution of different cDC subsets along the gastrointestinal tract was examined, from the duodenum to the terminal colon, characterizing both their phenotype and function. Our findings indicated that the cDC1 and CD103$^+$ cDC2 subtypes promote regulatory phenotypes in T cells via a RA–dependent mechanism (2,17); this tolerogenic profile is associated with the expression of PD-L1 in the small intestine, whereas in the colon, it appears to be dependent on XCR1 (18). These observations are consistent with the low expression of PD-L1 typically found in healthy human intestinal cDCs, with the exclusive intestinal CD103$^+$ cDC2 subset exhibiting the highest expression. This subset, when exposed to TGFβ, generates Tregs and shows an enhanced capacity to produce IL-10. Additionally, all cDC subsets (in contrast to monocytes) promoted the differentiation of IL-10 producing CD4$^+$ T cells.

Human intestinal CD103$^+$ cDC2 have emerged as a key tolerogenic DC subset in the gut. Recent human studies indicate that these CD103$^+$SIRPα$^+$ cDC2 express high levels of immunoregulatory molecules such as PD-L1 and produce anti-inflammatory IL-10 in the steady state (18,19). Indeed, mice lacking PD-L1$^+$ and XCR1$^+$ DC have a proinflammatory gut milieu associated with an increase in Th1/Th17 cells and a decrease in Treg cells and have exacerbated disease in the models of colitis (18). This work provides evidence that PD-L1$^+$ cDC2 in the small intestine may correspond to the CD103$^+$ cDC2 subset previously characterized as tolerogenic in both human and murine models. The enrichment of PD-L1$^+$ DCs in the duodenum, particularly within the SIRPα$^+$ cDC2 population, is in agreement with other studies performed in mice that showed that intestinal CD103$^+$ cDC2 exhibit a

tolerogenic phenotype marked by high PD-L1 expression, IL-10 production, and the capacity to induce regulatory T cells via RA and TGF-β (2,20,21). Given that CD103 expression is a hallmark of tolerogenic intestinal DCs, especially in the upper gut, it can be suggested that these PD-L1$^+$ cDC2 in mice represent the same functionally specialized CD103$^+$ cDC2 subset. Their compartmentalized distribution, with a dominant presence in the duodenum and decreasing abundance toward the colon, further supports their role in region-specific immune tolerance and suggests a functional specialization of cDC2 along the intestinal axis.

cDC efficiently migrate to mesenteric lymph nodes and induce gut-homing FOXP3$^+$ CD4$^+$ Tregs under the influence of the mucosal environment (19,22). In particular, CD103$^+$ cDC2 upregulate retinaldehyde dehydrogenase (RALDH) and use locally abundant TGF-β to generate RA, imprinting CCR9$^+$α4β7$^+$ gut-homing on differentiating Tregs (23,24). Consistent with a tolerogenic profile, human small intestinal cDC show especially high PD-L1 expression compared to colonic DC (18), and mTOR-dependent IL-10 production by these cDC2 is required to maintain gut homeostasis (19). Thus, human CD103$^+$ cDC2 combine high PD-L1 and IL-10 with RA/TGF-β–dependent signalling to potently drive FOXP3$^+$ regulatory T cell differentiation in the intestine (19). Supporting the idea of the tolerogenic role of CD103$^+$ cDC2, it has been also observed that CD103$^+$ cDC2 can acquire the ability to induce Th17 responses in the inflamed mucosa of CD patients. This shift from a tolerogenic to a pro-inflammatory profile suggests a functional plasticity of CD103$^+$ cDC2 and supports the hypothesis that, in CD, gut microenvironment make cDC to shift to a proinflammatory state and phenotype, losing their regulatory function and instead contributing to gut inflammation.

When studying these cells in mice, intestinal CD11b$^+$CD103$^+$ cDC2 are uniquely efficient at inducing FOXP3$^+$ Tregs (via a TGF-β and RA) (19,25). In contrast, intestinal CD103$^+$ cDC1 (XCR1$^+$) specialize in cross-presentation and Th1/Th17 immunity as they produce IL-12/IL-27 and primarily support IFN-γ producing T cells rather than classical CD4$^+$ Tregs (9,26,27). For example, cDC1-derived PD-L1, TGF-β and RA drive a FOXP3$^+$ regulatory CD8$^+$ T cell population in the gut (27), whereas cDC2 are the main inducers of FOXP3$^+$ CD4$^+$ Tregs (19). Reflecting these functional differences, murine cDC2 express high PD-L1 and ALDH in the small intestine (where they induce IL-13/TNF responses), whereas colon DC (enriched for XCR1$^+$) show lower PD-L1 and instead bias T cells towards IL-17/IL-22 programs (18). Thus, mouse models of intestinal immunity support that CD103$^+$ cDC2 carry a distinct tolerogenic signature (high PD-L1, IL-10/RA production, TGF-β dependence) relative to cDC1 or CD103- cDC2 (19,27).

On the other hand, although the proportion of colonic cDC2 does not decrease in IBD, colonic cDC2 from patients with IBD (both UC and CD) exhibit reduced expression of SIRPα a receptor implicated in the “don’t eat me” regulatory signal via its interaction with CD47 (28). Moreover, our study reveals a reduction in the cDC1 and CD103$^+$ cDC2 subsets in the inflamed colonic mucosa of UC patients, but not in CD. This differential distribution of cDC subsets suggests disease-specific alterations in mucosal immune regulation and highlights a potential divergence in the pathophysiological mechanisms underlying these diseases. The reduction of cDC1 in UC is particularly noteworthy given their role in maintaining intestinal homeostasis through cross-presentation and the induction of regulatory CD8$^+$ T cells, as well as their ability to produce IL-12 and IL-27, which contribute to the control of Th17-mediated inflammation (29,30). Their depletion could therefore exacerbate the dysregulated immune activation and epithelial barrier dysfunction that characterize UC. Similarly, the loss of CD103$^+$ cDC2 in the inflamed colon may further compromise mucosal immune regulation. This subset has been shown to induce Tregs via RA and TGF-β dependent mechanism (2,4), as well as to express immunoregulatory molecules such as PD-L1 and secrete IL-10, contributing to tolerance both in mice (18) and humans, as our observations showed. Their diminution in UC mucosa may reflect a failure of tolerogenic control, enhancing the persistence of proinflammatory responses.

In contrast, in CD, both cDC1 and CD103$^+$ cDC2 appear to be preserved in the inflamed tissue. However, their functional program may be altered. Indeed, it has been showed that CD103$^+$SIRPα$^+$ cDC2 isolated from the inflamed ileum of CD patients acquire the capacity to prime IL-17–producing CD4$^+$ T cells, suggesting a loss of regulatory function and acquisition of proinflammatory potential. This functional plasticity supports that, although tolerogenic DC subsets remain in CD tissue, they may shift their phenotype under inflammatory conditions. Conversely, in UC, the physical absence of these key regulatory DC subsets points to a more profound and possibly irreversible disruption of the tolerogenic network, potentially driven by differences in local cytokine environments, microbiota composition, or epithelial barrier cues (31–33). This divergence in cDC subset phenotype and function between UC and CD underscores the distinct immunological fingerprints of these diseases. It also highlights the potential of CD103$^+$ cDC2 and cDC1 as therapeutic targets, particularly in UC, where their depletion might represent a key player in disease progression. Therapeutic strategies aimed at preserving or restoring these populations could provide novel avenues to reestablish immune tolerance in the GI-tract of IBD patients. Further studies are needed to determine what factors trigger the change in cDC phenotypes and its dysfunction in UC, and whether these changes are reversible with treatment or immune-modulating interventions.

Previous studies have indicated that CD103$^+$ cDCs originate from CD103$^-$ cDC2 (25). This is also observed in our work, that showed an increased number of CD103$^+$ cDC2 after culturing LPMC. Notably, this differentiation process is inhibited by proinflammatory stimuli such as LPS, which promotes IL-1β secretion (although this inhibition is not exclusive to LPS). This inhibitory effect may mirror the scenario in IBD, wherein a proinflammatory microenvironment—that it was hypothesized that it is induced by microbiota stimuli—prevents cDC2 from differentiating into tolerogenic CD103$^+$ cDC2. This mechanism could explain the observed reduction in cDC1 and CD103$^+$ cDC2 within the inflamed colon of UC patients. Furthermore, although CD103$^+$ cDC2 expresses PD-L1 and is attributed with regulatory functions through IL-10 production and Treg stimulation, increased PD-L1 expression and a higher number of FOXP3$^+$ Treg cells have been observed in the intestinal mucosa of CD patients (34). This phenomenon may represent a compensatory mechanism by the immune system to mitigate inflammation or could be due to a malfunction in the PD-1/PD-L1 axis (35,36).

Taken together, our findings suggest a mechanism in the development of IBD in which inherently tolerogenic intestinal cDCs (which in normal conditions promote tolerogenic responses) encounter a proinflammatory stimulus—likely derived from the microbiota or its metabolites—resulting in a shift of the intestinal microenvironment towards a proinflammatory state. This shift reprograms cDCs, preventing their differentiation into a tolerogenic (CD103$^+$) profile and instead promoting the generation of IL-17A–producing Th17 cells.

Having showed the pivotal role played by cDCs in the immunological mechanisms underlying IBD, the hypothesis was that these cells might represent the therapeutic target for tofacitinib—a small molecule approved for the treatment of UC whose cellular targets are still not fully defined. To explore this hypothesis, a cytometry panel for the identification of human intestinal mAPCs was developed, encompassing the various subsets of cDCs, monocytes, and MΦs. This panel included markers of the JAK/STAT pathway (tofacitinib targets), as well as cytokines and receptors implicated in mucosal inflammation (such as CXCR3, TLR2, TLR4, IL-1β, IL-6, IL-10, TNFα and IL-15). Previous studies had demonstrated that tofacitinib could modulate the function of MO-DCs by reducing their stimulatory capacity (15,37). In contrast, our experiments using human intestinal cDCs isolated from intestinal resections—evaluated under basal conditions and following LPS stimulation in the presence/absence of tofacitinib did not show any change in their stimulatory capacity. This discrepancy may be explained by inherent differences between MO-DCs and human tissue-resident intestinal cDCs, as previously discussed. Thus, while tofacitinib may affect circulating DCs, it does not appear to impact tissue-resident intestinal

cDCs directly. Alternatively, tofacitinib might exert its therapeutic effect by suppressing proinflammatory T cell activation (13,38) without directly modulating the function of cDCs. However, an inhibitory effect of tofacitinib on intestinal human monocytes and MΦs was observed. Specifically, in UC patients (both active and quiescent), there was a reduction in JAK1 expression in monocytes, with a concomitant decrease in JAK1 in active UC monocytes and JAK3 in quiescent UC monocytes. This is, to the best of our knowledge, the first confirmation of the reduction in JAK1 and JAK3 levels in human intestinal monocytes and MΦs.

Previous studies have shown that tofacitinib can impair the function of MO-DCs by reducing their ability to stimulate T cells (15). These studies, largely based on *in vitro*–generated DCs from peripheral blood monocytes, support the notion that JAK-STAT signalling is crucial for DC maturation and proinflammatory cytokine production (39,40). However, our data, generated using human intestinal cDCs freshly isolated from surgical resections, presented a different scenario. Under both basal conditions and after stimulation with LPS, with or without tofacitinib, any significant changes in the allostimulatory capacity of intestinal cDCs were observed. This discrepancy may be due to intrinsic differences between MO-DCs and tissue-resident cDCs as have been explained above. These results suggest that while tofacitinib may modulate circulating myeloid cells, including MO-DCs, its direct impact on tissue-resident intestinal cDCs appears limited. Therefore, tofacitinib's therapeutic efficacy in UC may instead stem from its effects on downstream effector cells—particularly T lymphocytes. Supporting this, tofacitinib has been shown to suppress the differentiation and function of Th1 and Th17 cells by interfering with IL-2, IL-6, and IL-23 signalling pathways (13,38). These pathways are crucial for the expansion of pathogenic T helper subsets in IBD, suggesting an indirect mechanism by which tofacitinib may suppress inflammation. Interestingly, however, an immunomodulatory effect of tofacitinib on intestinal monocytes and MΦs was identified. In both active and quiescent UC patients, we observed a reduction in JAK1 expression in intestinal monocytes. Notably, JAK3 was selectively reduced in monocytes from quiescent UC patients. Prior studies have confirmed the inhibitory potential of tofacitinib on monocytes in systemic contexts by showing reduced phosphorylation of STAT5, a downstream target of JAKs that regulates proinflammatory cytokine transcription (41–43). Our findings extend these results to the human intestinal lamina propria and suggest a local anti-inflammatory mechanism mediated through disruption of JAK-STAT signalling in resident MΦs.

Additionally, an unsupervised analysis of the entire human intestinal myeloid APC compartment, enabled us to identify 16 distinct subsets, the majority of which corresponded to MΦs. Importantly, mAPCs from healthy controls more closely resembled those from

patients with quiescent UC than those from active UC, reinforcing the role of macrophage phenotypic plasticity in disease progression. However, treatment with tofacitinib did not significantly alter the proportional distribution of these subsets, indicating that while the drug effectively reduces JAK1 and JAK3 expression, it does not reprogram mAPC subset composition. This confirms that although tofacitinib downregulates JAK1 and JAK3 in monocytes and MΦs, it does not appear to modify the phenotype of these subsets.

8.2 MICROBIOME STUDY

Finally, given the central role of APCs in IBD, and having studied the differences between controls and IBD patients, it was ultimately decided to investigate the other major pillar in IBD research: microbiome. In this way, microbiota was evaluated, including bacteria and fungi (viruses were not possible to be assessed), in both human intestinal mucosa and stool (in this case, only bacteria). Initially, assessments of bacterial alpha and beta diversity did not reveal significant changes in either stool or intestinal mucosa samples. This contrasts with other studies that reported a decrease in bacterial diversity in IBD (44,45), particularly in active disease, which could be attributed to the low sample numbers per study group. Regarding fungal diversity in the intestinal mucosa, the literature presents different results: some described an increase (46), others a decrease in diversity in IBD (47), but all report a change in mycobiome (46–48). In our study, an increase in fungal diversity was observed in IBD patients in both faeces and intestinal biopsies, but it was not statistically significant.

Our analysis of the microbiota from both intestinal mucosal biopsies and stool samples from controls and IBD patients revealed a marked reduction in SCFA–producing bacterial genera in IBD, with a concomitant increase in genera with potential to contribute to disease under dysbiotic conditions. Among the most consistently reduced genera was Bacteroides, a major anaerobe in the healthy human gut known for its ability to produce acetate and propionate through fermentation of dietary fibers (49–51). The decline of *Bacteroides* in both CD and UC has been previously reported in numerous studies and is considered a hallmark of dysbiosis in IBD (52,53). SCFAs such as acetate, propionate, and butyrate are microbial metabolites known to maintain intestinal homeostasis by supporting epithelial barrier integrity, regulating mucosal immune responses, and promoting the differentiation of regulatory T cells (52,54). Thus, the loss of SCFA-producing microbes in IBD may facilitate inflammation and mucosal damage. Moreover, our data showed an increase in potentially harmful genera, such as *Fusobacterium*, which has been associated with pro-inflammatory activity, epithelial invasion, and colorectal carcinogenesis (55–57). The expansion of *Fusobacterium* in the inflamed gut likely contributes to the disruption of epithelial integrity and perpetuation of chronic inflammation in IBD (55). Interestingly, not all SCFA-producing genera were uniformly decreased. In stool samples, *Agathobacter*, a known butyrate producer, was significantly reduced during active disease but showed increased abundance in remission phases, suggesting a dynamic response of this genus to the inflammatory environment. Similarly, mucosal biopsies from IBD patients showed an increase in genera such as *Faecalibacterium* and *Alistipes*. *Faecalibacterium prausnitzii* is a key butyrate producer with documented anti-inflammatory properties (58), and its

increase in certain mucosal areas may represent a compensatory attempt by the microbiota to restore homeostasis. Conversely, the increased presence of *Alistipes* and *Turicibacter* in IBD tissues may reflect more complex and context-dependent roles. While *Alistipes* has been associated with both anti-inflammatory and pro-inflammatory effects depending on the host condition and microbial community structure (59), *Turicibacter's* role remains less clear but has been linked to host serotonin signalling and mucosal immunity (60). These findings underscore the need to consider tissue localization and disease phase when interpreting microbiota shifts.

Altogether, our results highlight a complex restructuring of the intestinal microbial ecosystem in IBD, characterized by a loss of beneficial SCFA-producing bacteria and an expansion of potentially pathogenic or opportunistic genera. Importantly, the differential patterns observed between stool and mucosal samples reinforce the value of assessing both luminal and mucosa-associated microbiota, as they may represent distinct ecological niches with varying implications for disease activity.

The intestinal microbiota plays a pivotal role in maintaining immune homeostasis, primarily through the production of SCFAs, including butyrate, acetate, and propionate. These metabolites are known to promote Treg differentiation and sustain a tolerogenic environment in the gut by modulating the function of local antigen-presenting cells, especially DCs (61–63). In our study, both mucosal and faecal samples from IBD patients revealed a reduction in SCFA-producing genera, such as *Bacteroides*, *Agathobacter*, and *Roseburia*, particularly during active disease, consistent with previous findings (64,65). In contrast, an enrichment of potentially human pathogenic taxa was observed, including *Fusobacterium*. Moreover, certain SCFA-producing genera such as *Faecalibacterium* and *Alistipes*, which may have context-dependent effects, were found to increase in mucosal biopsies despite their reduction in faeces, suggesting spatial differences in microbial function and interaction with the host immune system (66). This microbial dysbiosis has significant implications for the function of intestinal cDCs. Mechanistically, SCFAs such as butyrate act as histone deacetylase (HDAC) inhibitors, leading to downregulation of costimulatory molecules (CD80, CD86), MHC-II, and proinflammatory cytokines like IL-12, while enhancing IL-10 production, thus promoting Treg differentiation over inflammatory subsets (67). In addition, SCFAs bind to G-protein-coupled receptors such as GPR109A and GPR43 expressed on DCs, further reinforcing anti-inflammatory responses (68,69); activation of GPR109A by butyrate has been shown to induce Tregs and suppress Th17 responses in colitis models (70). These regulatory mechanisms have been mainly described in murine models, but human data support similar effects: butyrate-treated human MO-DCs exhibit decreased CCR7 and IL-12 expression and promote IL-10-

producing T cells instead of Th1 or Th17 cells (71). Therefore, the depletion of SCFA-producing bacteria observed in IBD patients may impair the capacity of intestinal cDCs to acquire tolerogenic functions while instead promote Th17 responses (72–74). Thus, the interplay between microbial dysbiosis and local inflammation likely disrupts the tolerogenic capacity of cDCs, reinforcing a feedback loop that perpetuates chronic inflammation and mucosal immune dysregulation in IBD.

Comparing the bacterial composition of the intestinal mucosa with that of stool samples revealed significant differences in microbial profiles. These results highlighted distinct patterns between mucosal and luminal communities, underscoring the influence of local microenvironmental factors, such as oxygen gradients, host immune interactions, and nutrient availability, on shaping the microbiota composition in each compartment (75,76). Despite these differences, a substantial overlap in bacterial taxa was observed, with many genera and species being shared between tissue biopsies and faecal samples. This overlap suggests that stool samples may serve as a practical and informative window into the mucosal microbial landscape. However, it is important to know that faecal samples do not fully capture the mucosa-associated microbiota, particularly those bacteria that preferentially colonize the epithelial surface or exist in close contact with host cells (77,78). Consequently, while faecal microbiome analysis remains a valuable non-invasive tool for studying gut microbial dynamics, it should be interpreted as an approximation rather than a complete representation of the mucosal ecosystem. This distinction is particularly relevant in IBD, where mucosal dysbiosis may be more closely associated with disease (64,74).

In terms of fungal composition, although no statistically significant differences were observed in alpha and beta diversity metrics between IBD patients and controls, an increased number of fungal genera was detected in the IBD groups. This observation is consistent with previous reports suggesting that dysbiosis in IBD also affects the mycobiome (79). Interestingly, the genera enriched in IBD samples in our study were not the well-characterized *Candida* or *Saccharomyces*, which have historically been associated with mucosal inflammation and immune activation in IBD (80,81), but rather lesser-known fungal genera whose role in gut immunity remains poorly understood. This highlights a critical gap in our understanding of the intestinal mycobiome and calls for more studies characterizing these underrepresented genera in the context of intestinal inflammation. Additionally, a reduction in *Saccharomyces* in IBD patients was shown compared to controls, which aligns with findings linking decreased abundance of this genus to gut barrier dysfunction and impaired anti-inflammatory responses (47,82). Specifically, *S. boulardii* can promote a tolerogenic DC phenotype by downregulating proinflammatory

cytokines (e.g., IL-6, IL-12, TNF-α) and enhancing the secretion of anti-inflammatory mediators such as IL-10, thereby contributing to the expansion of Tregs and the maintenance of immune homeostasis (83). Additionally, *S. boulardii* has been shown to preserve epithelial barrier function by enhancing the expression and localization of tight junction proteins, such as occludin and zonula occludens-1 (ZO-1), and by reducing epithelial permeability in models of inflammation-induced barrier dysfunction (84). Given these immunomodulatory and barrier-stabilizing properties, the observed decrease in *Saccharomyces* in patients with IBD may contribute to a shift toward a more proinflammatory intestinal microenvironment, thus exacerbating disease pathogenesis. However, due to limited sample size and the high degree of interindividual variability observed in fungal community composition, it was not possible to define a consistent dysbiotic signature. Further studies with larger, well-stratified cohorts and integrative multi-omics approaches will be necessary to clarify the role of the fungal microbiota in the pathogenesis and progression of IBD.

It is important also to highlight that diet -one of the most influential modulators of the gut microbiota- was not controlled in this study. Dietary intake can significantly alter the composition and metabolic activity of the intestinal microbiota, thereby influencing immune responses and disease activity in IBD patients (85,86). However, due to its high variability and the difficulty of accurately monitoring dietary habits over time, this factor was not incorporated into our analyses.

Having described both microbiome and mycobiome, the potential regulatory interactions within the microbiota has been also investigated. The correlation analysis of the intestinal mucosal microbiota revealed a complex network of interactions between bacterial and fungal genera that may reflect regulatory dynamics within the ecosystem, particularly in the context of IBD. Notably, certain fungal genera such as *Wallemia* showed positive correlations with commensal, SCFA–producing bacteria like *Roseburia*, and negative correlations with proinflammatory (in human health context) genera such as *Fusobacterium*. This is consistent with the hypothesis that specific fungi may contribute to the maintenance of microbial homeostasis through their interactions with beneficial bacteria.

Interestingly, a greater number of bacteria–fungi correlations were described during remission phases of IBD compared to periods of active inflammation. Many of these associations involved SCFA-producing bacteria and fungi with less characterized or potentially regulatory roles. This observation aligns with findings from a study which demonstrated that microbial network disturbances are more pronounced during active CD,

whereas remission phases exhibit more stable and interconnected microbial communities (87). Other study revealed alterations in the fungal microbiota of IBD patients, suggesting that changes in fungal–bacterial inter-kingdom relationships may influence disease activity (47). Collectively, these findings suggest that increased microbial network complexity and stability during remission may reflect compensatory mechanisms that favour immune tolerance and tissue repair. It is also interesting that during active phases of IBD, correlations were dominated by potentially pathogenic taxa such as *Fusobacterium, Escherichia/Shigella*, and fungal genera with known proinflammatory properties, which may reflect a breakdown of regulatory cross-talk and a shift toward microbial configurations that sustain inflammation (88). In remission state, it was described that beneficial cross-kingdom correlations—such as positive associations between *Wallemia* and SCFA-producing bacteria like *Roseburia* and *Agathobacter*—were more prominent, suggesting a cooperative and potentially immunoregulatory ecosystem. This pattern is consistent with other studies using multi-omics profiling to show that remission in IBD is associated with more complex and stable microbial co-occurrence networks, including bacteria–fungi interactions (89). Altogether, these findings support the concept that interkingdom microbial interactions are central modulators of gut immune homeostasis, especially in the context of IBD. This agrees with studies that support that fungal and bacterial species in the gut do not operate in isolation but instead engage in complex ecological networks that can either sustain immune tolerance or exacerbate inflammation depending on the health status of the host (90). These condition-dependent microbial interactions underscore the importance of viewing IBD not only as a disease of bacterial dysbiosis but also one of disrupted trans-kingdom symbiosis.

The identification of reliable, non-invasive biomarkers is a critical goal in the management of IBD, particularly to support diagnosis, monitor disease activity, and predict therapeutic responses. In this context, stool samples offer a practical, cost-effective and patient-friendly alternative to mucosal biopsies, and the presence of microbial signatures in faeces that reflect mucosal conditions holds considerable clinical promise. Based on our comparative analyses of microbial profiles in mucosal biopsies and paired stool samples, three bacterial genera with biomarker potential were identified: *Prevotella, Fusobacterium*, and *Roseburia*, each exhibiting disease- and phase-specific patterns across intestinal compartments.

Prevotella was consistently elevated in both stool and mucosa during active IBD, supporting its potential as a biomarker of disease flare. Although traditionally associated with fiber-rich diets and considered part of a “healthy” enterotype in some populations, certain *Prevotella* species have been implicated in proinflammatory responses through TLR2 activation and promotion of Th17 differentiation (91). The expansion of *Prevotella* in

active IBD may therefore signal mucosal immune activation and microbial imbalance. *Fusobacterium*, particularly *F. nucleatum*, was enriched in stool and mucosal samples from patients with active CD (92,93). This genus has been previously associated with proinflammatory properties, epithelial barrier disruption, and increased disease severity in CD (56,92). Its invasive potential and strong correlation with inflamed mucosa further support its utility as a biomarker specific to CD activity. Moreover, *Fusobacterium* has shown predictive value in other intestinal diseases such as colorectal cancer, which agrees with the clinically relevancy taxon for inflammation-associated pathology. Finally, *Roseburia*, a well-known SCFA-producing genus with anti-inflammatory properties, was more abundant in stool and mucosa during quiescent UC and reduced during active disease. Its capacity to produce butyrate suggests a role in maintaining remission. Further longitudinal studies are needed to confirm their potential use as biomarkers.

The integration of taxonomic, functional, and correlation-based microbiota data from both mucosal and stool samples provides new insights into the complex ecological dynamics underlying IBD. Our findings reveal not only a reduction in beneficial SCFA-producing taxa and an enrichment of proinflammatory microorganisms in active disease but also highlight the importance of interkingdom microbial interactions, particularly the regulatory roles of underexplored fungal genera, in modulating immune responses. Moreover, the consistent presence of genera such as *Prevotella*, *Fusobacterium*, and *Roseburia* across compartments support their value as faecal biomarkers for disease type and phase. These results reinforce the concept that intestinal dysbiosis in IBD is not merely a consequence but an active participant in disease pathogenesis, capable of reshaping immune cell phenotypes and functions. Unravelling these networks will be crucial for the understanding of IBD pathogenesis and in the long term could support the development of microbiota-based therapies aimed at restoring immune and microbial homeostasis in patients with IBD.

All the described changes in microbiome and mycobiome composition implies also a reduction in compounds such as SCFAs that are needed to maintain intestinal homeostasis and, consequently, these microbial alterations are likely to influence the intestinal microenvironment disrupting immune tolerance. The hypothesis is that the dysbiotic state, characterized by the depletion of SCFA-producing bacteria and expansion of potentially pathogenic species, creates a microenvironment that impairs the tolerogenic function of intestinal cDC. Specifically, exposure to proinflammatory microbial products and altered metabolic signals such as reduced SCFA may drive cDCs to lose their regulatory phenotype (marked by expression of molecules like PD-L1 and production of IL-10) and instead adopt a proinflammatory profile. This shift in cDC, particularly in gut specific $CD103^+$ cDC2 phenotype could promote Th1/Th17-mediated inflammation, triggering the chronic

intestinal inflammation characteristic of IBD. Therefore, the combined dysbiosis and impaired cDC tolerogenicity likely form a feed-forward loop that sustains mucosal inflammation and tissue damage in IBD. This interplay between the microbiota and immune system highlights new avenues for therapeutic intervention, including microbiota modulation aimed at restoring beneficial microbial populations and reinforcing cDC-mediated immune tolerance. The identification of microbial biomarkers such as *Prevotella*, *Fusobacterium*, and *Roseburia* offers potential tools for disease monitoring and new treatment strategies.

8.3 LIMITATIONS AND STRENGTHS

This study presents several limitations that must be described, as well as significant strengths that contribute to improve our understanding of intestinal immunology and its associated microbiota in the context of IBD. One of the main limitations lies in the low number of samples used for functional assays, primarily due to the inherent difficulty in obtaining intestinal resections from patients with CD, and even more so from patients with UC, since surgical interventions are much less frequent in UC. This restricts our capacity to functionally study the function of the different subsets of intestinal cDCs, particularly in the context of UC. Nevertheless, one of the strengths of our approach is the use of human intestinal cDC, which are difficult to isolate due to the low proportion that they represent in the total LPMC. In most studies, these cells are analyzed within the total population of LPMCs, often identified by flow cytometry gating strategies without being physically separated, or replaced by monocyte-derived DCs. In contrast, our ability to isolate and study *bona fide* intestinal DCs enhances the physiological relevance of our findings and expands current knowledge regarding the role of these APC in mediating inflammatory processes within the human gut. Another important consideration is the nature of the control intestinal samples, which were obtained from individuals undergoing colonoscopy due to non-specific gastrointestinal complaints. Although their mucosa appeared macroscopically normal, we cannot totally discard the presence of subclinical or molecular alterations that could influence our results. Additionally, the cDCs used as controls in the T cell immunostimulation assays were isolated from the healthy tissue adjacent to resected intestinal tumors. Although this tissue is not macroscopically inflamed, the proximity to the tumor may influence local immune responses, potentially altering DC phenotype or function. Despite this, we successfully isolated and analyzed distinct subsets of human intestinal cDCs, co-culturing them with allogeneic T cells to assess their stimulatory capacity. This allowed us to delineate the specific roles of each subset in promoting regulatory or inflammatory responses, providing novel insights into their functional specialization in the intestinal microenvironment.

Another limitation of the study is the patient treatment status. Recruiting IBD patients not undergoing any therapy is challenging, especially in cases of clinical remission, which often depends on ongoing pharmacological intervention. Consequently, most patients included in cDC subset characterization and the evaluation of the effect of tofacitinib over APC were under treatment, primarily with azathioprine and mesalazine, both of which are known to influence DC biology. Azathioprine (AZA) and its metabolite mercaptopurine (6-MP) have been shown to impair DC maturation and activation by downregulating costimulatory molecule expression and reducing pro-inflammatory cytokine production, while promoting

Treg expansion (94). These agents also reduce DC migration, thereby limiting their ability to prime T cells (95). Methotrexate (MTX), another common immunosuppressant, has similar effects by suppressing DC activation, reducing IL-12 and TNF-α levels, and enhancing IL-10 production (96). Moreover, MTX can induce oxidative stress and apoptosis in DCs, thereby reducing their viability and immunogenic potential. While these pharmacological effects may confound some of the immunological readouts, all patients included in our study were confirmed to have active mucosal inflammation through endoscopic scoring—SES-CD for CD and Mayo Endoscopic Score for UC—ensuring that our analyses captured immune activity in the context of ongoing intestinal inflammation.

In the microbiological component of the study, a notable limitation was the low number of samples in each patient cohort, as well as the absence of fungal diversity and characterization data from stool samples. Despite these constraints, one of the major strengths of our approach was the parallel analysis of bacterial communities in both fecal and mucosal samples from the same individuals. This allowed for a more comprehensive understanding of the gut microbiota and its spatial variation and provided valuable insights into the potential utility of faecal biomarkers for disease monitoring and diagnosis. Finally, an important technical limitation was encountered in the viral metagenomic analysis of intestinal biopsy samples. Although *shotgun* sequencing was performed, the high proportion of human DNA in these samples severely compromised viral DNA detection, ultimately precluding a reliable analysis of the intestinal virome.

8.4 FINAL SUMMARY OF REMARKABLE FINDINGS

In summary, this study has optimized a robust protocol for isolating human intestinal LPMCs from biopsies and resections, allowing for the comprehensive identification and characterization of distinct APC subsets, including cDC1, cDC2, $CD103^+$ cDC2, monocytes, and MΦs. Notably, it was observed that intestinal cDCs play a pivotal role in maintaining immune tolerance via mechanisms involving PD-L1, and that their functional reprogramming in an inflammatory environment -probably due to microbiota dysregulation- contributes to the pathogenesis of IBD by shifting from a tolerogenic to a proinflammatory profile. Moreover, while tofacitinib did not modulate the function of tissue-resident cDCs, it significantly reduced JAK1 and JAK3 expression in monocytes and MΦs, revealing different cellular responses. Complementarily, our investigations into the intestinal microbiota revealed notable alterations in bacterial and fungal compositions associated with IBD, identifying potential non-invasive biomarkers such as *Prevotella*, *Fusobacterium*, and *Roseburia*, and highlighting complex interkingdom correlations that may regulate intestinal homeostasis. Collectively, the findings presented in this thesis provide insights into the immunopathogenesis of IBD and identifies potential immune fingerprints, enhancing our understanding of the disease and laying the groundwork for the development of more precise therapeutic strategies and non-invasive diagnostic tools.

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Chapter 9. Conclusions

1. We have successfully optimized a protocol for the isolation of human intestinal cDCs and macrophages from surgical resections, allowing the implementation of a reproducible workflow to work with human intestinal tissue avoiding the use of monocyte-derived DC or mouse cDC.
2. We have defined both the phenotype and function of the different cDC subsets in the human intestine in health and disease, proving that:
    - Different cDC subsets exhibit differential spatial distribution along the intestinal tract.
    - $CD103^+$ cDC2 cells are potentially tolerogenic in the gastrointestinal (GI)-tract of controls.
    - All cDC subsets in control tissue promote T cell responses with a tolerogenic profile, characterized by IL-10 production.
    - cDC1 and $CD103^+$ cDC2 subsets are reduced in the inflamed tissue from patients with active UC
    - In the inflamed ileum from CD patients, $CD103^+$ cDC2 cells promote the proliferation of $IL\text{-}17^+$ helper T cells.
3. In IBD, particularly in inflamed tissues, $CD103^+$ cDC2 cells lose their tolerogenic properties, contributing to the breakdown of immune tolerance.
4. Through unsupervised analysis, the profile of APCs from the intestinal mucosa of qUC patient resemble those of controls more closely than those of patients with active disease.
5. Tofacitinib does not significantly affect the phenotype or function of cDCs. However, it modulates the activity of monocytes and macrophages by reducing the expression levels of JAK1 and JAK3.
6. We have successfully characterized the bacterial (bacteriome) and fungal (mycobiome) components of the intestinal mucosa in both control and IBD patients. IBD samples showed a marked reduction in short-chain fatty acid (SCFA)-producing species, especially butyrate producers, along with altered fungal community composition.
7. Fecal and mucosal bacterial profiles differ significantly, even within paired samples from the same individuals, in both control and IBD cohorts.

8. Microbial correlation analysis revealed that, in controls, potentially beneficial bacteria are positively associated with beneficial fungi and negatively associated with pathogenic fungi. In contrast, during active IBD, these correlations weaken or reverse, and positive associations between bacterial and fungal pathogens emerge.
9. Based on our findings, we propose three potential fecal biomarkers for IBD disease states: *Prevotella* for active IBD, *Fusobacterium* for active Crohn's disease, and *Roseburia* for quiescent ulcerative colitis.