Universidade do Minho
Escola de Medicina

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Deciphering the microbiome and metabolic factors contributing to protection against ulcerative colitis

Tese de Doutoramento em Envelhecimento e Doenças Crónicas

Trabalho efetuado sob a orientação do
Doutor Ricardo Jorge Leal Silvestre
e do
Doutor Duarte Custal Ferreira Barral

dezembro de 2018

STATEMENT OF INTEGRITY

I hereby declare having conducted my thesis with integrity. I confirm that I have not used plagiarism or any form of falsification of results in the process of the thesis elaboration.

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ABSTRACT

Inflammatory bowel disease (IBD) is a complex set of inflammatory disorders from the gastrointestinal tract, comprising Crohn's disease (CD) and ulcerative colitis (UC). Although the precise etiology of this disease remains unclear, the pathogenesis of IBD has been defined as an immune-mediated condition with a strong genetic predisposition, triggered by environmental factors that affect the mucosal barrier and the balance of the gut microbiota. In this work, we aimed to identify relevant microbiota species that may influence the susceptibility or protection against colitis development and how the modulation of dietary nutrients impacts the outcome of disease.

While analyzing dextran sulfate sodium (DSS)-induced colitis in genetically similar C57BL/6 mice housed in two different animal facilities, we serendipitously observed a group of animals with a remarkable protection to disease development. The two groups of mice display distinct microbiota and metabolic profiles, clustering separately in multivariate data analysis. This can be at the genesis of the resistant phenotype, since fecal microbiota transplant (FMT) from resistant to susceptible mice was able to reverse colitis susceptibility. We identified *Akkermansia muciniphila* and *Parabacteroides distasonis* as metagenomic top hits, being enriched in resistant mice, and in susceptible mice after receiving FMT from the resistant group. Resistant mice also evidenced significantly increased levels of IL-10, IL-17 and IL-22, as well as increased expression of claudin- and mucin-encoding genes in homeostatic conditions when compared to the susceptible group, suggesting that the protective phenotype is associated with an intestinal epithelial barrier more prone to sustaining an inflammatory insult. Indeed, by analyzing the effect of these bacterial candidates on colonic epithelial cells using a biochip-based human gut model, an increased expression of E-cadherin was found when cells were incubated with *A. muciniphila*, recapitulating the findings observed *in vivo*. We are currently dissecting in the gut-on-a-chip model how these candidates modulate epithelial barrier integrity to increase protection against an inflammatory event, to further validate these results in an *in vivo* susceptible model of colitis.

In parallel, we were focused in evaluating how dietary supplementation can contribute to the protection against the development of IBD or, at least, for a better resolution of the disease. It is known that the metabolic environment in the gut is altered during pathology. Therefore, we analyzed the colon metabolic profile of mice during colitis development, in order to identify possible altered nutrients that might be used as promising prophylactic or therapeutic

approaches. We observed that mice treated with DSS display a twofold decrease in threonine levels in the intestine. Knowing this, threonine supplementation was evaluated as possible therapy by treating mice on the beginning of colitis development or when the inflammation was already established. We observed that threonine has a detrimental effect when administered during colitis onset, with mice evidencing a delayed remission of the disease when compared to control group or mice treated with threonine only during inflammation. This detrimental effect is associated with a reduction in the number of goblet cells per crypt and also in decreased amounts of IL-22 in the gut.

Overall, the findings presented in this thesis contribute to a better understanding of the different factors that can modulate protection and/or susceptibility to IBD development, providing novel insights to possible strategies of tackling IBD.

RESUMO

A doença inflamatória intestinal (DII) é um conjunto de distúrbios inflamatórios do trato gastrointestinal que engloba a doença de Crohn e a colite ulcerosa. Embora a etiologia desta doença ainda não seja clara, a patogénese da DII é uma condição mediada por respostas imunes, com uma forte predisposição genética, desencadeada por fatores ambientais que afetam a barreira da mucosa e o equilíbrio do microbiota intestinal. Este trabalho tem como objetivos a identificação de espécies do microbiota que possam influenciar a suscetibilidade ou proteção contra o desenvolvimento de colite e de que forma é que a modulação de nutrientes presentes na dieta afeta o decurso da doença.

Ao analisar o perfil de colite induzida por dextrano sulfato de sódio (DSS) em murganhos C57BL/6 geneticamente semelhantes e alojados em diferentes biotérios, observamos por acaso que um dos grupos de animais apresentava uma elevada proteção contra o desenvolvimento da doença. Os dois grupos de murganhos apresentam perfis metabólicos de microbiota distintos, agrupando-se isoladamente na análise de dados multivariada. Estes perfis podem estar na base do fenótipo de proteção, visto que o transplante de microbiota fecal (FMT) de animais resistentes para suscetíveis foi capaz de reverter a suscetibilidade à colite. Através de uma análise de metagenómica, identificamos que as espécies *Akkermansia muciniphila* e *Parabacteroides distasonis* encontram-se significativamente enriquecidas em murganhos resistentes ou em animais suscetíveis após FMT proveniente de murganhos resistentes. Estes animais também apresentaram níveis significativamente elevados de IL-10, IL-17 e IL-22, bem como um aumento na expressão de genes que codificam as proteínas claudinas e mucinas, em condições de homeostasia quando comparados ao grupo suscetível, o que sugere que o fenótipo protetor está associado à existência de uma barreira epitelial intestinal mais propensa a tolerar um insulto inflamatório. Aliás, através da análise das bactérias candidatas em células epiteliais do cólon usando um modelo de intestino humano em formato biochip, foi detetada uma maior expressão de E-caderina nas células incubadas com *A. muciniphila*, recapitulando as observações feitas *in vivo*. Neste momento estamos a explorar no modelo "gut-on-a-chip" de que forma esses candidatos modulam a integridade da barreira epitelial para aumentar a proteção contra um evento inflamatório, para validar posteriormente esses resultados num modelo *in vivo* de suscetibilidade à colite.

Em paralelo avaliamos de que forma a suplementação na dieta contribui para a proteção contra o desenvolvimento de DII ou, pelo menos, para uma melhor resolução da doença. O ambiente metabólico no intestino é alterado durante a patologia. Como tal, analisamos o perfil metabólico do cólon de murganhos durante o desenvolvimento de colite, com vista a identificar possíveis nutrientes alterados que possam ser utilizados em estratégias de profilaxia ou terapia. Murganhos tratados com DSS exibiram uma diminuição de duas vezes nos níveis de treonina no intestino. Como tal, a suplementação com treonina foi avaliada como possível terapia, sendo administrado este nutriente a murganhos no início do desenvolvimento de colite ou quando a inflamação já estava estabelecida. Descobrimos que a treonina tem um efeito prejudicial quando administrada durante o início da colite, com os animais a evidenciar uma recuperação mais lenta da doença quando comparados com o grupo controlo ou com murganhos que receberam treonina apenas durante a inflamação. Simultaneamente a este efeito prejudicial, encontrou-se uma redução no número de células caliciformes por cripta intestinal e níveis mais baixos de IL-22 no intestino.

Em conclusão, os resultados apresentados nesta tese contribuem para uma melhor compreensão dos diferentes fatores que podem modular a proteção e/ou suscetibilidade ao desenvolvimento de DII, dando novas perspetivas sobre as possíveis estratégias para combater esta doença.

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LIST OF ABBREVIATIONS

5-ASAs	5-Aminosalicylates
AB	Alcian blue
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
AOM	Azoxymethane
APC	Allophycocyanin
ATG16L1	Autophagy-related protein 16-1
BCA	Bicinchoninic Acid
BDL	Below Detection Level
BHI	Brain Heart Infusion
BIR	Baculovirus Inhibitor of apoptosis protein Repeat
BLAST	Basic Local Alignment Search Tool
BV	Brilliant Violet
CARD	Caspase Activation and Recruitment Domain
CCR2	C-C chemokine receptor type 2
CD	Crohn's Disease
CD	Cluster of Differentiation
CDH	Cadherin
cDNA	complementary Deoxyribonucleic acid
CFU	Colony-Forming Units
CLDN	Claudin
CoA	Coenzyme A
CX3CR1	CX3C chemokine Receptor 1
DAI	Disease Activity Index
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic Cell
DII	Doença Inflamatória Intestinal
DMEM	Dulbecco's Modified Eagle's medium
DNA	Deoxyribonucleic acid
DSS	Dextran Sulfate Sodium

ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal Bovine Serum
FDR	False Discovery Rate
FITC	Fluorescein isothiocyanate
Flt3L	FMS-like tyrosine kinase 3 ligand
FMT	Fecal Microbiota Transplant
FOXP3	Forkhead box P3
FSC	Forward Scatter
GATA3	GATA-binding protein 3
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GPR43	G-Protein coupled Receptor 43
GWAS	Genome-Wide Association Studies
HBSS	Hank's Balanced Salt Solution
HCEC	Human Colonic Epithelial Cells
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HIF	Hypoxia-Inducible Factor
HUVECs	Human Umbilical Vein Endothelial Cells
IBD	Inflammatory Bowel Disease
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate Lymphoid Cells
IRF3	Interferon-Regulatory Factor 3
IRGM	Immunity-related GTPase family M protein
kDa	kilodalton
KLF4	Krüppel-like Factor 4
ko	knockout
LPL	Lamina Propria Leukocyte
LRR	Leucine-Rich Repeat
LTi	Lymphoid Tissue-inducer

MAPK	Mitogen-Activated Protein Kinase
M-CSF	Macrophage Colony-Stimulating Factor
MHC	Major Histocompatibility Complex
MLCK	Myosin Light Chain Kinase
MMPs	Matrix MetalloProteinases
moMØ	monocyte-derived Macrophages
MUC	Mucin
MyD88	Myeloid Differentiation primary response 88
NACHT	NAIP, CIITA, HET-E and TP1
NAIP	Baculoviral IAP repeat-containing protein 1
ND	Not Detected
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NK	Natural Killer
NLRP	NOD-like Receptor family Pyrin domain
NMR	Nuclear Magnetic Resonance
NOD	Nucleotide Oligomerization Domain
NS	Not Significant
OCLD	Occludin
OD	Optical Density
OTU	Operational Taxonomical Unit
PAS	Periodic Acid-Schiff
PBS	Phosphate-Buffered Saline
PCoA	Principal Coordinate Analysis
PE	Phycoerythrin
PE-Cy7	Phycoerythrin-Cyanine 7
PerCP-Cy5.5	Peridinin Chlorophyll Protein Complex-Cyanine 5.5
PET	Polyethylene terephthalate
PLS-DA	Partial least squares Discriminant Analysis
PMA	Phorbol Myristate Acetate
PRRs	Pattern Recognition Receptors
PYD	Pyrin Domain
qRT-PCR	quantitative Real-time Polymerase Chain Reaction

RAG1	Recombination Activating Gene 1
RELMβ	Resistin-like Molecule Beta
RICK	Receptor-interacting serine/threonine Kinase
RNA	Ribonucleic acid
ROR	Retinoic acid-related Orphan Receptor
Rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
rRNA	ribosomal Ribonucleic acid
SCFA	Short-Chain Fatty Acid
SCID	Severe Combined Immunodeficiency
SIMCA	Soft Independent Modelling of Class Analogies
SPF	Specific-Pathogen-Free
SSC	Side Scatter
STAT	Signal Transducer and Activator of Transcription
Tbet	T-box transcription factor
TGF-β	Transforming Growth Factor beta
Th	T helper
TIR	Toll/Interleukin-1 Receptor
TLRs	Toll-like Receptors
TNBS	Trinitrobenzene Sulfonic Acid
TNF-α	Tumor Necrosis Factor alpha
Treg	regulatory T
TRIF	TIR-domain-containing adapter-inducing Interferon-β
Ubq	Ubiquitin
UC	Ulcerative Colitis
ZO	Zonula Occludens

CHAPTER I

GENERAL INTRODUCTION

1. INFLAMMATORY BOWEL DISEASE

Inflammatory bowel disease (IBD) is a complex set of chronic relapsing inflammatory disorders that affect the gastrointestinal tract. It comprises two main clinical forms: ulcerative colitis (UC), in which the mucosal inflammation is circumscribed to the colon; and Crohn's disease (CD), in which transmural inflammation may occur throughout the gastrointestinal tract. IBD usually emerges in the second and third decade of life, with a significant percentage of the patients progressing to a relapsing chronic disease [1]. Although the precise etiology of this disease remains unclear, both forms of IBD seem to result from an inappropriate inflammatory response triggered by intestinal microbes in genetically susceptible individuals [1-3]. IBD has an extensive impact in patients' quality of life due to the early onset, the subsequent morbidity due to the fluctuations of the disease and the absence of an efficient therapy.

1.1. Prevalence and incidence of IBD

Traditionally considered a disease of western countries, the epidemiology of IBD has been changing around the world during the last decades. With the rise of newly industrialized countries in Asia, Africa and South America, IBD became a global disease, affecting mainly young adults and being associated to the adoption of a more westernized lifestyle [4].

The prevalence of IBD finds its higher values in developed areas such as North America, Europe (especially northern and western countries), Australia and New Zealand, with almost 200 to 300 cases per 100 000 people for CD, and 300 to 500 cases per 100 000 for UC (Figure I.1) [4]. In these countries, IBD is related to higher rates of morbidity and mortality, as well as a significant economic burden for health care systems. An estimated 2.5 to 3 million individuals are affected with IBD in Europe, having a direct cost of 4.6 to 5.6 billion euros per year [5].

While in developing countries the prevalence of IBD is significantly lower when compared to developed ones, the incidence of the disease, i.e. the rate of new or newly diagnosed cases, has rapidly risen in the last three decades, which anticipates a global increase in the number of IBD patients (Figure I.2; [4]). Several studies have pointed out alterations in the geographical distribution of UC, with an increase in the number of reported cases in Southeast Asia and Latin America [6, 7].

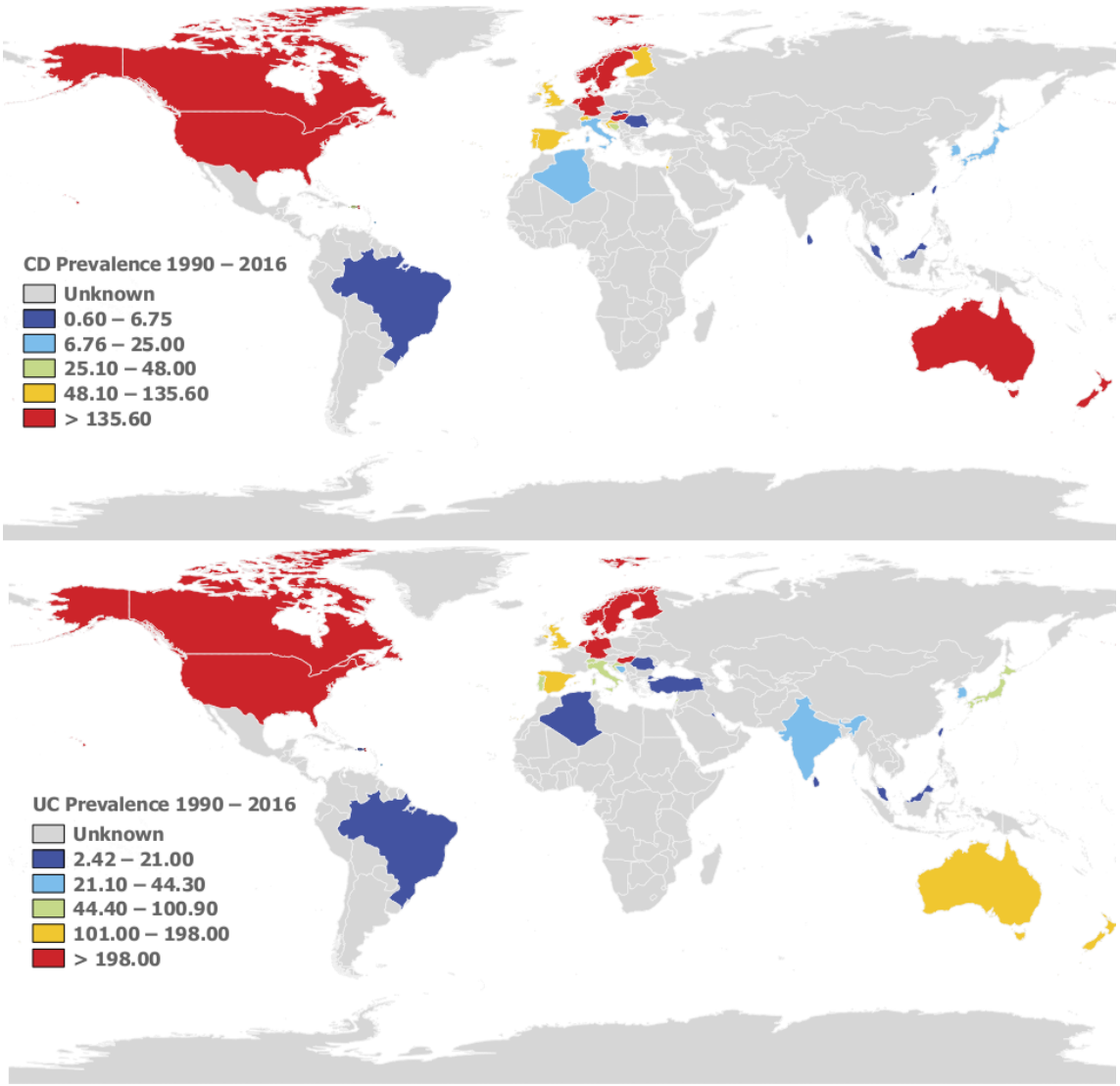

Figure I.1. Worldwide prevalence of Crohn's disease (CD) and ulcerative colitis (UC) between 1990 and 2016. Data from Ng *et al.* [4].

Despite having a common ground, UC and CD present a distinct distribution among men and women. While data regarding gender prevalence of UC are not fully conclusive, with some studies stating that UC is more prevalent in men, while others mentioning no major differences in both genders, it is estimated that CD occurs more frequently in females [6, 8]. The different access to health care and the presence of risk factors, genetic or environmental, may explain in some extent the divergent incidence of IBD among different populations [9]. Epidemiological studies focused on south Asian immigrants in the United Kingdom have shown that this population display a similar incidence rate of UC than the local one, when in their countries of origin the disease prevalence in considerably lower, thus supporting the argument that environmental factors can be paramount for the development of the disease [10]. Hence, the

exacerbated inflammation that is on the basis of IBD is defined by a combination of genetic and environmental factors that subsequently impact the immune response.

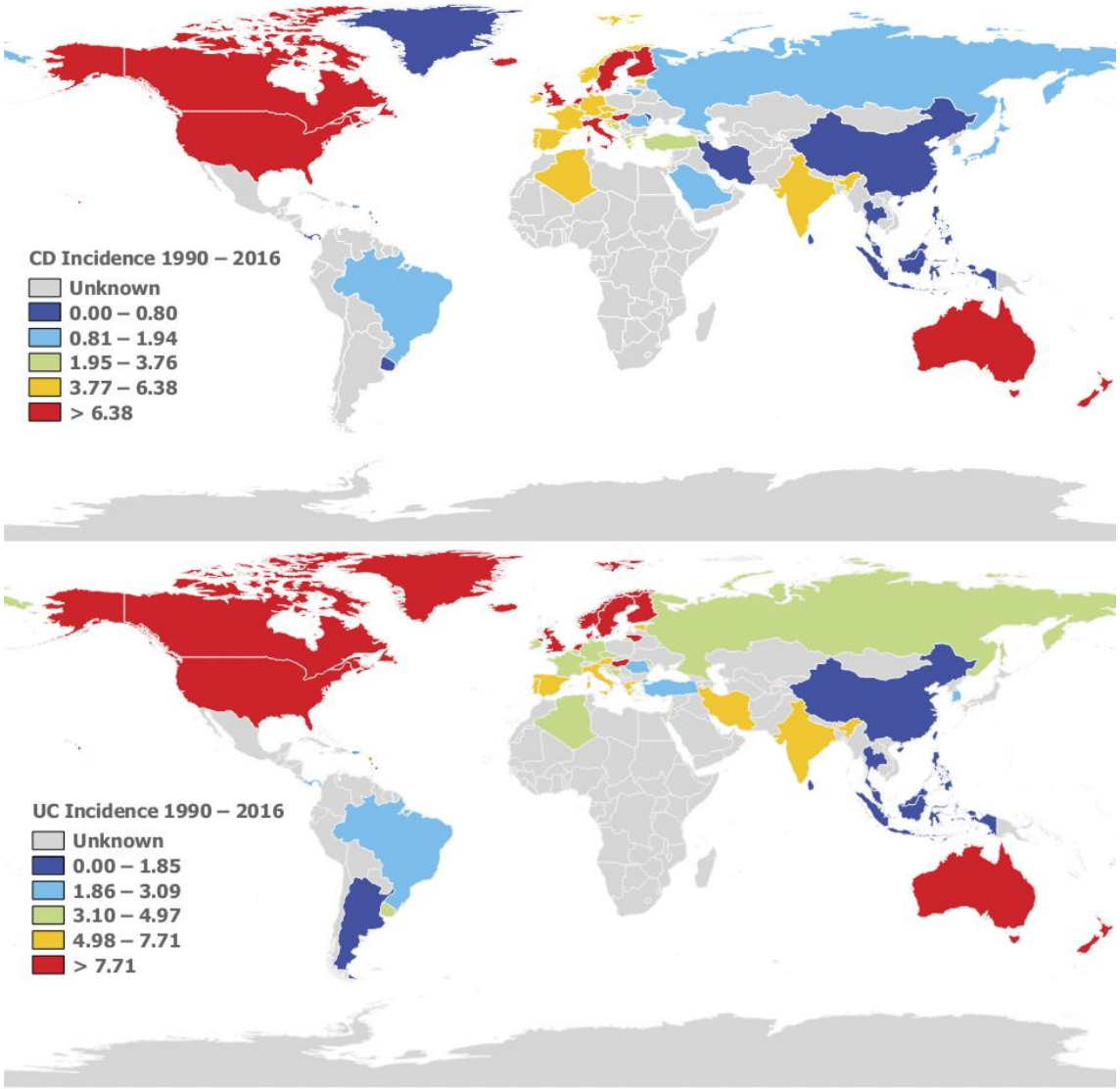

Figure I.2. Worldwide incidence of Crohn's disease (CD) and ulcerative colitis (UC) between 1990 and 2016. Data from Ng *et al.* [4].

1.2. Etiology of IBD

The diversity of clinical presentations and response to treatments reinforces the idea that IBD is not a single disease, but a spectrum of inflammatory disorders from the gastrointestinal tract whose etiology is not well defined. Several genetic associations have already been described within CD and UC, namely in genetic regions containing nucleotide-binding oligomerization domain 2 (*NOD2*) and autophagy genes such as autophagy-related protein 16-1 (*ATG16L1*) (that has been associated with CD but not, so far, to UC), pointing towards a common genetic basis in

both disorders; notwithstanding, the development of IBD is mostly driven by immune mechanisms [2, 11, 12]. In addition, environmental factors such as diet and lifestyle can be involved in the proneness to intestinal inflammatory diseases. For instance, smoking habits affects inversely these two disorders, since smokers are at increased risk for CD, as well as more severe manifestations of the pathology, whereas in UC this behavior is associated to protection [2, 6]. The dissimilarity between UC and CD, together with the divergent efficacy of treatment between patients (it is known that up to one-third of the CD patients undergoing anti-tumor necrosis factor alpha (TNF-α) treatments do not respond properly to therapy [13]), evidences the need of targeted and personalized therapies in order to maximize the benefits for the patients. Overall, this spectrum of disorders needs to be deeply characterized, aiming for the development of new therapeutic adjuncts to effectively tackle IBD.

1.3. Symptoms and diagnosis

The symptoms associated to IBD are variable according to the severity of the disease, as well as the localization of the inflammation. Systemic symptoms, such as weight loss and fever, are often found in CD patients and also in severe cases of UC [14, 15]. Although in CD any portion of the gastrointestinal tract can be affected, the presence of inflammation in the ileum is more frequent. This disorder can be patchy and segmental with transmural inflammation, contrary to what is observed in UC [1]. CD can be classified according to the extensiveness of the disease in five categories. Ileocolitis is the most prevalent form of CD and affects the terminal part of the small intestine (ileum) and the colon, whose symptoms are mainly diarrhea and cramping, along with significant weight loss; ileitis, in which only the ileum is affected and has similar manifestations as ileocolitis; jejunoileitis is characterized by inflammation in upper half of the small intestine, leading to intense abdominal pain, fistula formation and diarrhea; gastroduodenal CD affects both the stomach and the beginning of the small intestine (duodenum) and leads to loss of appetite, nausea, vomiting and weight loss; and Crohn's (granulomatous) colitis, in which only the colon is affected, presenting a UC-like clinical phenotype which includes diarrhea, rectal bleeding and can frequently lead to the formation of fistulas and ulcers in the anus [16, 17].

The most common symptom in UC patients in the presence of blood in the stool, which is reported to occur in more than 95% of the cases of active disease [14]. The presence of superficial and diffuse lesions is very common, with deep ulceration only observed in severe pathology [1, 6, 18]. The colon and the rectum are affected by the inflammation, which can

progress in a continuous retrograde way along the colon. Based on the affected area of the colon, UC can be classified in four distinct subcategories: ulcerative proctitis, in which inflammation in limited to the rectum – and for this reason it tends to be a milder form of UC; proctosigmoiditis, in which both the rectum and the sigmoid colon (lower segment of colon above the rectum) and it is characterized by bloody diarrhea, tenesmus and moderate pain; left-sided colitis, that encompasses inflammation from the rectum to the splenic flexure, and in which is often the occurrence of diarrhea, severe pain on the left side of the abdomen, bleeding and weight loss; and pan-ulcerative colitis, which affects the entire colon and common manifestations include diarrhea, bleeding, severe abdominal pain, extensive weight loss and cramps [19, 20]. Moreover, one-third of UC patients develop extraintestinal manifestations of colitis, such as arthritis, primary sclerosing cholangitis, uveitis and psoriasis [21, 22].

1.4. Current treatments and novel therapies for IBD patients

Although there is no medical cure for IBD, several therapies have been proven to effectively contribute for the control of the disease in most of the cases, helping the induction and maintenance of remission and subsequently improving the patients' quality of life. Aminosalicylates, corticosteroids and immunomodulatory agents are frequently prescribed to IBD patients as first-line of treatment, with the anti-TNF-α therapy often used as a frequent choice for IBD management [23-26].

5-Aminosalicylates (5-ASAs), such as mesalazine and sulfasalazine, are commonly used in therapy for patients with UC, but have shown to be ineffective to resolve the clinical symptoms and tissue inflammation in CD [27]. The efficacy of these compounds in UC pathology is associated with a reduction of prostaglandin synthesis via inhibition of cyclooxygenase, blockade of neutrophil chemotaxis, suppression of proinflammatory cytokines and impairment of nuclear factor-κB activation (NF-κB) in immune cells [28]. 5-ASAs are able to induce and maintain UC remission and also to decrease the risk of developing colitis-associated cancer in UC patients [29].

Corticosteroids, such as budesonide and prednisone, have also a broad application in the induction of remission in patients with UC or CD, despite the lack of efficiency in maintaining the disease in this state [16, 18]. Other immunosuppressive drugs, such as azathioprine, methotrexate and 6-mercaptopurine, are included as IBD therapy [30-32]. Nevertheless, these therapeutic adjuncts are usually combined with other therapies, such as anti-TNF-α.

Anti-TNF-α therapy was approved by the United States Food and Drug Administration (FDA) for use in CD in 1998 and for UC in 2005 [23, 25]. The emergence of this type of therapy was mainly due to the fact that a significant number of IBD patients were intolerant or did not respond properly to the classic agents previously discussed. Infliximab, adalimumab, and certolizumab pegol, as well as other biosimilars, are some of the anti-TNF agents used in the clinic to induce and maintain remission of the disease in both CD and UC patients [16]. Some of the anti-TNF agents, such as infliximab, are administered in combination with immunosuppressive agents, given that clinical trials demonstrated their capacity to promote clinical remission when compared to monotherapy in both CD and UC [31, 33]. The intravenously administration of infliximab is also used for several other inflammatory conditions such as psoriasis and rheumatoid arthritis [34, 35]. TNF-α is produced by a myriad of immune and non-immune cells in the gut, such as macrophages, dendritic cells (DCs), T lymphocytes and fibroblasts [36], and it has pleiotropic effects in the intestine, such as regulation of T cell apoptosis, activation of macrophages to the production of proinflammatory cytokines and regulation of matrix metalloproteinases (MMPs) by fibroblasts in the resolution of tissue damage [37, 38]. Thus, by inhibiting the binding of TNF-α to its receptor, it is possible to overturn intestinal inflammation through several pathways.

Patients who develop complications, such as abscesses or malignancy, or do not respond properly to treatment, need to undergo surgery in order to oppose the disease. Moreover, responsiveness to medical therapy, fulminant colitis, perforation of the colon, massive bleeding, dysplasia-associated lesions and cancer are other events that can lead to surgical intervention, which normally consists in the removal of the affected area [18]. It is estimated that 5 to 20% of the patients with UC face colectomy [39-41]. Although it can be effective in some UC cases, it is not curative for CD patients. Nevertheless, complications can derive from the process, such as small intestine obstruction, fistulas and persistent pain, which strikingly impacts quality of life and leads to severe morbidity [42, 43].

The fact that there are still patients who do not respond to the currently available therapeutic approaches, or in whom the side effects derived from the therapy do not enable the treatment to continue, emphasizes the need of developing new drugs and personalized strategies for IBD management. Vedolizumab is one of the most recent options available in the market as alternative drug to treat IBD. Approved for treatment of both moderate-to-severe cases of CD and UC in May 2014 (both by the US FDA and the European Commission), vedolizumab is a α4β7-

integrin-specific antibody that has shown efficacy in inducing and maintaining remission in CD and UC [27, 44]. It acts by selectively block intestinal lymphocyte trafficking, without impacting the traffic to the central nervous system, thus suppressing inflammation [45]. Other drugs targeting proinflammatory cytokines were tested, such as anti-interferon (IFN)-γ (fontolizumab) and anti-interleukin (IL)-17A (secukinumab), without substantial efficacy in disease resolution [27, 46]. Ustekinumab, a monoclonal antibody to the p40 subunit of IL-12 and IL-23, was recently approved as therapy for CD in Europe and the USA [47].

The manipulation of intestinal microbiota by probiotic administration has also been explored as possible therapy to IBD. Studies focused on the effects of colonization with the non-pathogenic Gram-negative bacteria *Escherichia coli* Nissle 1917 have demonstrated that this strain is able to impact intestinal barrier function and leads to the production of anti-inflammatory molecules. This strain stimulates β-defensin production by intestinal epithelial cells and enhances tight junction formation by the upregulation of zonula occludens proteins [48, 49]. Fecal microbiota transplantation (FMT) has also been pointed out as a possible strategy to combat IBD and even other pathologies associated with intestinal dysbiosis. Several studies have shown the efficacy of this method in the treatment of *Clostridium difficile* infection, suggesting a potential use of FMT to promote the remission of UC in patients [50-54]. More recently, it was described the first case series of immune-checkpoint inhibitors-associated colitis that were successfully treated by FMT from healthy donors [55]. Nevertheless, a deeper comprehension regarding the different factors that contribute to the development of the disease and the identification of specific biomarkers that facilitate a differential diagnosis is essential to develop more adequate and personalized therapies to tackle IBD.

2. THE MICROBIOME AND MUCOSAL IMMUNITY

2.1. The intestinal environment

The human intestine is a complex ecosystem that harbors a diverse and vastly evolved microbial community composed by several distinct species. It is estimated that the gut contains approximately 100 trillion microbial organisms, such as fungi, archaea, virus and mostly bacteria, which are collectively known as the gut microbiota. These symbiotic microbial species have coevolved with the host, establishing mutualistic relationships and influencing the evolutionary fitness of the host through their genome and proteome and as well as by the production of metabolites, constituting altogether the intestinal microbiome [56-58].

The composition and diversity of the gut microbiota within the host varies throughout life, with several bacterial species occupying specific niches or being related with a particular growth phase of the individual (Figure I.3) [59]. The first event shaping the composition of the gut microbiota occurs during birth, with the transmission of several species from the mother to the neonate. Indeed, several studies have shown that the pioneer microbial heritage from the mother to the neonate depends on if it is a vaginal or a caesarian-section delivery [60-62]. Even though the species acquired from the first contact are dissimilar, the microbial composition converges over the first years of life, which demonstrates that the gut microbiota evolves throughout life to a state of homeostasis in which the resident species are massively adapted for survival in such competitive environment [63].

The gut microbiota is mainly composed by species of the Bacteroidetes phylum during childhood, but in adult life there is a shift between the Bacteroidetes and Firmicutes species, with the latter being the most represented phylum [59]. In adults, approximately 99.9% of the cultivatable bacterial populations are obligate anaerobes, with the genera *Bacteroides*, *Clostridium*, *Lactobacillus*, *Fusobacterium*, *Bifidobacterium*, among others, being highly abundant. The number and diversity of species also increases from the small intestine to the colon [58]. These microbial populations play beneficial roles in maintaining the regular physiology and homeostasis of the host, for example, by the defense against opportunistic pathogens, the supply of essential nutrients, the digestion of otherwise indigestible compounds such as plant polysaccharides, and by their role in the development and regulation of the immune system [58, 64, 65].

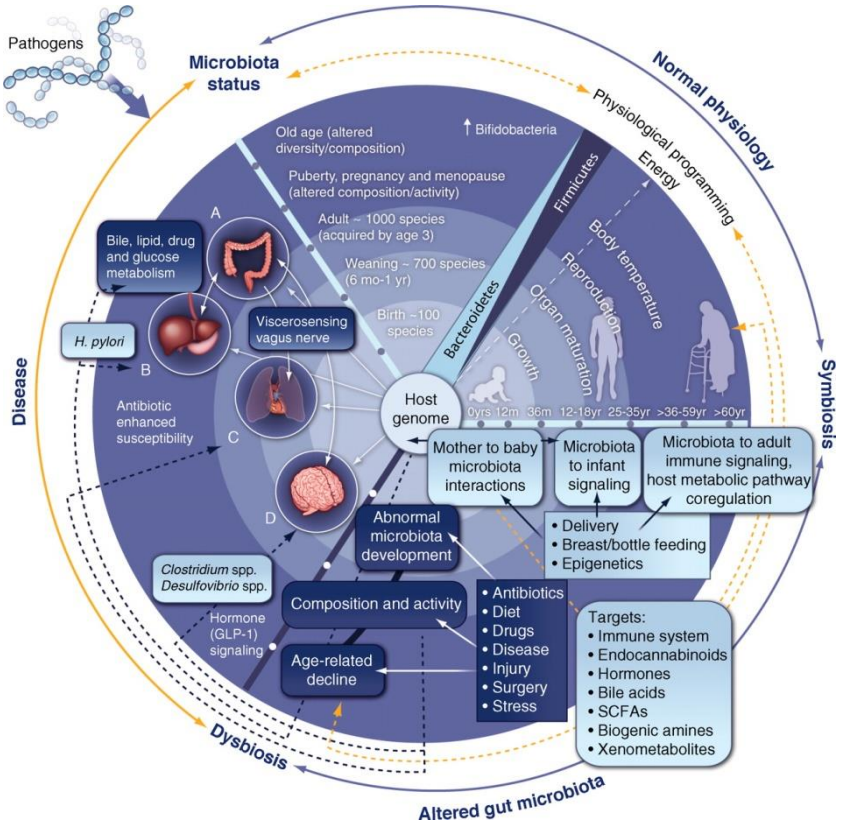

Figure I.3. Gut microbiome dynamics throughout human development. Maternal microbiota is inherited by the newborn. Several factors, such as nutrition and genetics, influence the development of a healthy intestine and alterations in the early life can predispose for disease development in the future. Gut microbiota composition shifts from more abundance of Bacteroidetes to increased Firmicutes species throughout life. The gut microbiota modulates the host physiology and impairments in its composition may lead to several pathologies such as IBD, cancer and obesity. From Nicholson *et al.* [59].

Host genetics and environmental factors, such as nutritional habits, lifestyle, the occurrence of diseases and antibiotic use, are able to impact the composition of microbial communities throughout life, by influencing the diversity and functioning of the gut microbiota and impacting human health [66-68]. In fact, dysbiosis, i.e., the imbalance within the microbial communities, is associated with the development of several diseases, namely IBD [69], cancer [70], diabetes [71], allergy [72] and obesity [73]. Hence, the understanding on how intestinal bacteria can shape the host immune system is decisive in order to elucidate the etiology of inflammatory diseases and subsequently tailor strategies for prevention and treatment.

The pathogenesis of IBD has been defined as an immune-mediated condition with a strong genetic predisposition, generated by environmental factors affecting the mucosal barrier and the balance of the gut microbiota. Once the intestinal epithelial and mucosal barriers are

compromised, bacteria from the luminal side are able to reach the lamina propria, breaking the tolerance of immune cells to intestinal microbes and exerting an activation of the immune cells by the direct contact with the bacterial products. This ultimately can lead to the elimination of the infiltrating bacteria, or to an inappropriate immune response that can culminate in tissue damage, which will maintain the activation of immune cells and lead to the development of IBD [36].

2.2. Intestinal epithelial barrier-mediated homeostasis

The epithelial barrier of the intestine represents the largest interface between the internal organs and the environment. This barrier allows the free exchange of water, electrolytes and nutrients between the intestinal lumen and the underlying tissues while simultaneously enclosing the microbial populations and antigens present in the lumen milieu [74, 75]. The proper integration of the diverse internal and external stimuli by the epithelial barrier is essential to preserve intestinal homeostasis.

The central component of the intestinal epithelial barrier is the epithelial cell layer, which corresponds to the luminal surface of the small intestine and colon. This barrier is composed by a single layer of columnar intestinal epithelial cells responsible for the maintenance of a healthy intestinal environment. The epithelial barrier needs to accurately allow a selective permeability by transepithelial/transcellular and paracellular pathways. The first route is mainly associated with the transport of solutes through the epithelial cells and controlled by selective transporters for electrolytes, short-chain fatty acids (SCFAs), sugars and amino acids. On the other hand, paracellular permeability is involved in the transport through the epithelial intercellular space and is coordinated by intercellular complexes localized in the apical-lateral membrane junction [75, 76].

A proper barrier function is mostly dependent on the regulation of the paracellular transport. This is mediated by a series of intercellular junctions, such as tight and adherens junctions as well as desmosomes [77]. While adherens junctions and desmosomes are primarily involved the adhesive forces necessary for cell-cell communication via cadherin interaction, tight junctions are the main players for paracellular permeability [74]. The tight junction network displays both size and charge selectivity routes of paracellular efflux named pore and leak pathways. The former is a high-capacity, size-selective and charge-selective route that is mostly determined by the expression of claudins, which are transmembrane tight junction proteins that

are able to form pores to regulate ion selectivity. Oppositely, the leak pathway is a low-capacity route with more limited selectivity that is mainly regulated by zonula occludens (ZO)-1, occludin and myosin light chain kinase (MLCK) [78].

Claudins are 20- to 27-kD integral membrane proteins containing four hydrophobic transmembrane domains, two extracellular loops, and N- and C-terminal cytoplasmic domains, and they display specific tissue and cellular expression patterns [75]. Occludin is a tetraspanning integral membrane protein with two extracellular loops, a short cytoplasmic N-terminus and a long cytoplasmic C-terminus [79, 80]. These proteins are expressed mostly at tight junctions in epithelial and endothelial cells, but also in other cell types such as neurons and DCs, and they participate in the regulation of paracellular permeability [81, 82]. These proteins play a key role in the regulation of epithelial cell motility and several studies have described impaired expression of claudin-3, -5 and -8 and occludin in patients with CD [83], as well as down-regulation of claudin-1 and -4 and increased expression of claudin-2 in UC patients [84].

Upon epithelial injury, tight junctions are degraded and the transport occurs by the unrestricted pathway, which is high-capacity and non-selective, allowing bacteria and microbial antigens cross the barrier and subsequently leading to an inflammatory event [85]. This pathway was demonstrated to be predominant in dextran sulfate sodium (DSS)-induced colitis in mice and also in patients with necrotizing enterocolitis [86]. The relevance of the intestinal epithelial barrier for IBD predisposition is supported by the observation of abnormal intestinal permeability and, as previously mentioned, alterations in the expression of tight junction proteins in IBD patients. In fact, intestinal permeability was addressed as a prognosis marker due to its increase during CD remission [87-89].

Additionally to columnar epithelial cells, the intestinal barrier is also composed by the presence of specialized cells along the crypt villi. Goblet cells are an essential partner in gut homeostasis, since they are responsible for the production and secretion of mucin glycoproteins that constitutes the mucus layer, which prevents the direct contact of luminal microorganisms with the epithelial layer [3, 90]. Goblet cells are more abundant in the colon when compared to the small intestine, which is also proportional with the higher number of bacteria present in the colon. The mucus layer is constituted by inner and outer fractions of mucin 2, an *O*-glycosylated protein [91]. The inner fraction is attached to epithelial cells and free from intestinal bacteria. The outer and loose mucus fraction harbors a large amount of microorganisms that can perform the proteolytic processing of polysaccharides of mucin as energy source. Thus, the absence of

dietary fibers, that are the main energy source of gut bacteria, may lead to an increased expansion of mucin-degrading species and increased processing and/or degradation of the mucus layer [91, 92]. Reports have shown that Resistin-like molecule-beta (RELMβ), a specific protein of goblet cells, is induced upon bacterial colonization and that its transcription impairment decrease the severity of experimental DSS-induced colitis [93]. Moreover, experiments performed in MUC2-deficient mice, which lack the gene encoding for mucin 2 were shown to develop spontaneous colitis [94]. Consistent with these findings are also other observations that demonstrate that intestinal mucus is paramount for the suppression of colitis [95].

Paneth cells are also important components of the epithelial barrier due to the secretion of antimicrobial peptides [2]. These cells are mainly found in the small intestine being absent in the colon. The reduced amount of goblet cells in the small intestine when compared to the colon highlights the extremely important role of Paneth cells for the segregation of intestinal bacteria and epithelial cells. Antimicrobial peptides released by these cells are small, amino acid-rich cationic proteins that interact with the negatively charged microbial cell membrane and leads to membrane disruption. They can be classified into α-, β- and θ-defensins, in which α-defensins are the most relevant to fight pathogenic bacteria [96]. Several studies have described that a decrease in α-defensins produced by Paneth cells may be detrimental to intestinal balance and may contribute to the pathogenesis of terminal ileal CD in patients with mutations in the *NOD2* gene [97, 98]. Therefore, these findings support the central role of intestinal epithelial barrier in the maintenance of intestinal homeostasis and that slight imbalances in its regulation may lead to the onset of intestinal inflammation.

2.3. Recognition of microbes by the host

Intestinal epithelial cells act as a physical and chemical barrier between the lamina propria, which is highly inhabited by immune cells, and the lumen, where commensal microbes reside. When this barrier is compromised, immune cells encounter a plethora of microorganisms that become exposed [74]. This poses an enormous challenge to the immune system since it needs to be able to properly respond to pathogens without mounting an inflammatory response that may be detrimental to commensal populations and may lead to the development of spontaneous inflammation.

Intestinal epithelial cells express in their surface receptors for diverse chemokines and pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) and NOD proteins. PPRs can be expressed by immune and non-immune cells in the intestinal mucosa, such as macrophages, DCs and epithelial cells [99]. TLRs are the first to detect the presence of pathogens, driving cellular responses through the secretion of several signaling molecules, such as MyD88 (pathway shared by all TLRs with the exception of TLR3) and TIR domain-containing adapter protein inducing IFN-β (TRIF). This leads subsequently to the activation of mitogen-activated protein kinase (MAPK) signaling, the transcription of NF-κB and also interferon-regulatory factor 3 (IRF3) and upregulation of genes encoding for proinflammatory cytokines [99, 100].

Several TLRs have a prominent role during pathogen infection. For instance, the activation of TLR2 and TLR4 is fundamental for the elimination of the pathogen during *Salmonella enterica* subsp. *enterica* serovar Typhimurium [101]. It was demonstrated that mice lacking TLR2, TLR4 or both receptors display increased bacterial burden in the spleen and mesenteric lymph nodes upon infection when compared to wild-type mice [101, 102]. TLR2-deficient mice also evidenced alterations in barrier function and severe colitis when infected with *Citrobacter rodentium* or *Campylobacter jejuni* [103, 104]. Moreover, TLR2-mediated recognition of curli fibrils in the gut seems to contribute to protection, since the recognition of *Salmonella* spp. curli fibrils triggers inflammatory response and promotes epithelial barrier function, which demonstrates the relevance of TLR signaling both during pathogen infection and in the maintenance of mucosal integrity [105].

How it is possible for the immune system to discriminate between commensals and pathogens it still not clear, even more knowing that TLR ligands are present on both commensal and pathogenic microbes. This may be partly explained by the fact that cytosolic PRRs are not directly in contact with commensal bacteria and also because, in intact epithelium conditions, some cell membrane-bound PRRs are mostly expressed on the basolateral side of polarized intestinal epithelial cells and not on the apical side that is exposed to luminal antigens. In case of disruption of the epithelial barrier, by inflammation or pathogenic infection, it is allowed the binding to the basolateral TLRs and the initiation of the inflammatory response [106].

NOD-like receptors are divided in three main categories, based on their amino-terminal domain: the CARD-containing nucleotide-binding oligomerization domain proteins or NOD proteins, such as NOD1 and NOD2; the NACHT-, LRR- and PYD-domain-containing proteins, such

as NOD-like receptor family pyrin domain (NLRP) 3 and NLRP6; and BIR-containing proteins (or NAIPs) [107]. In the intestine, Paneth cells express NOD2 and epithelial cells express both NOD1 and NOD2. These receptors identify fragments of peptidoglycans, such as muramyl dipeptide, present in both Gram-positive and Gram-negative bacteria, that is recognized by NOD2 [108, 109]. After ligand recognition, NOD1 and NOD2 interact with the receptor-interacting serine/threonine kinase (RICK), which in turn will lead to the activation of NF-κB [110].

These receptors have shown so far an important role during infection. For instance, NOD2 is known to take part on the immune response against *C. rodentium* in mice. In fact, it was shown that mice lacking NOD2 evidenced 10- to 100-fold more *C. rodentium* in the feces in comparison to wild-type upon infection [111]. NOD1 and NOD2 are also relevant to control infection with *Salmonella* spp. and *Shigella* spp., by interacting with RICK and activating MAPK and NF-κB pathways, and therefore triggering the inflammatory response [112, 113].

NLRP4 receptor also aids to restore intestinal homeostasis by contributing to the elimination of enteric pathogens and by protecting the host from intestinal carcinogenesis, since it is able to identify, among the epithelial layer, cells that have undergone harmful damage [114, 115]. NRLP6 is modulated by the levels of polyamines and amino acids that are present in the gut and it regulates host-microbe interactions by the production of inflammasome-mediated IL-18 and the downstream expression of antimicrobial peptides and also the secretion of mucus by goblet cells [116, 117]. Indeed, the absence of NLRP6 has a striking impact on the gut microbiota composition and function and in the increase susceptibility to enteric infections, which supports the idea that NRLP6 plays a key role in the regulation of microbiome in the gut [118, 119].

2.4. Regulation of mucosal homeostasis by immune cells

The intestinal lamina propria and epithelium harbor the largest population of immune cells in the entire body. The lamina propria is populated by several immune cells such as T cells, B cells, macrophages and DCs. These cell populations are players in a complex net of immune mechanisms that aim to maintain intestinal homeostasis, being prepared to respond to any harmful stimuli that may arise in the intestinal environment [120].

2.4.1. Innate immunity

2.4.1.1. Myeloid cells

Mononuclear phagocytes are innate immune cells involved in the uptake and presentation of antigens in the gut. Within this subset, intestinal macrophages are the most abundant population in the body and also the largest population of leukocytes in the intestine, which confers them the role of first line of defense to maintain intestinal homeostasis [121]. Most adult tissue macrophages are originated during embryonic development and not from circulating monocytes. However, several studies have established that body's barrier tissues, such as skin, lungs and intestine are continuously replenished by blood-derived monocytes that differentiate *in situ*. Circulating monocytes comprise multiple subsets, defined by their phenotype, morphology and transcriptional profiles, and can be distinguished by the expression of CD14 and CD16 in humans and Ly6C, C-C chemokine receptor type 2 (CCR2) and CX3C chemokine receptor 1 (CX3CR1) in mice [122]. Full monocyte maturation as well as the acquisition of a characteristic functional signature by gut mucosa macrophages is essential for the maintenance of intestinal homeostasis [123]. The majority of intestinal macrophages are located in the colon, which is directly associated with the amount of bacteria present in the gut [124].

Ly6C^{hi} monocytes constitutively enter the intestinal mucosa and differentiate via a CX3CR1 intermediate stage into mature CX3CR1^{hi}F4/80^{+} cells that are localized in the vicinity of the epithelium. Here, they constitutively produce IL-10, an anti-inflammatory cytokine that is crucial for the maintenance of intestinal homeostasis and for innate and adaptive response against intracellular pathogens. IL-10 is important not only to balance inflammation by blocking proinflammatory responses derived from TLR stimulation [125, 126], but also because this cytokine promotes the survival and function of FOXP3^{+} regulatory T (Treg) cells in the gut [127]. Intestinal macrophages also help to maintain the integrity of the gut epithelial barrier, having several other essential functions, including phagocytosis and degradation of microorganisms and dead tissue cells and the production of mediators that promote epithelial cell renewal [120, 128].

The monocyte-to-macrophage differentiation seems to be impaired when Ly6C^{hi} monocytes respond to microenvironmental factors and commensal microbiota, becoming proinflammatory and therefore having a key role in the pathogenesis of IBD. Multiple factors may contribute to the functional and phenotypical characteristics acquired by monocytes during their differentiation process in the gut [121, 123]. The strongest evidences come from experimental animal models,

which have shown that mice with impaired IL-10 signaling or a monocyte/macrophage-specific deletion of IL-10 receptor present a disruption of the monocyte differentiation pattern observed in the steady state of wild-type mice, leading to spontaneous development of severe colitis [129, 130]. Similar disturbances were detected in the monocyte-to-macrophage populations of human gut mucosa during UC and CD [131], whose risk increases with impaired IL-10 production or polymorphisms in the *IL10* locus [132]. Therefore, the absence of IL-10 signaling is, in part, responsible for an inappropriate monocyte-to-macrophage differentiation, resulting in the impairment of mucosal homeostasis and development of inflammatory diseases.

Small intestine and colonic macrophages express major histocompatibility complex (MHC) class II and CX3CR1, and no major functional differences have been described for both macrophage groups. Hence, it seems that similar features must control the development and functions along the intestine [126, 133, 134]. Notably, intestinal macrophages are sedentary and do not migrate to the mesenteric lymph nodes, thus unlikely influencing the priming of naïve T cells. Nevertheless, they are able to interact with these cells, leading to secondary expansion of the newly arrived, primed T cells in the gut mucosa and subsequently contributing to the mounted immune response [128].

Distinguished from intestinal macrophages by the lack of CD64 and F4/80 expression, other prominent mononuclear phagocyte population in the intestine consists in DCs. DCs are the most effective antigen-presenting cells and a major link between innate and adaptive immune response. These cells present antigens to and activate T cells, decisively impacting the type of immune response [135]. Intestinal DCs derive from a FMS-like tyrosine kinase 3 ligand (Flt3L)-dependent precursor and develop into several distinct subsets, based on the expression of CD11b and CD103 (also known as integrin αE): $CD103^+CD11b^-$ DCs, $CD103^+CD11b^+$ DCs and $CD103^-$ DCs [136]. CD103-expressing DCs are particularly important in the maintenance of gut homeostasis since they are needed for the imprinting of gut homing phenotypic alterations on antigen-specific T cells and for the induction of tolerogenic immune responses in the intestine [137]. The role of $CD103^+$ DCs in the intestine is controversial, because they have a relevant role in Th17 cell differentiation and induction of immune responses, but also are associated with immune regulatory activities [138]. $CD103^+CD11b^+$ DCs are present in different amounts alongside the gut and in association with the presence of Th17 cells, which may indicate a putative role of this DC subset in the regulation of Th17 cell homeostasis [139]. This is supported by reports that described a reduction in Th17 cells after selective depletion of $CD103^+CD11b^+$

DCs in mice [140]. On the other hand, it is also known that $CD103^+$ DCs aid in the generation of Treg cells from naïve T cells, with $CD103^+CD11b^-$ and $CD103^+CD11b^+$ subsets apparently displaying a redundant role in the maintenance of Treg numbers [135, 141]. In turn, CD103- cell subset, although representing a minor DC population, can promote Th1 or Th17 cell responses and are described to contribute to enhanced colitis in mice [142, 143].

Although the exact mechanism by which intestinal DCs keep homeostasis in the gut are still unclear, growing evidence has demonstrated that DCs can interact with the gut flora and modulate itself in terms of immune regulatory function. For example, the secreted mucin 2 by goblet cells has the ability to imprint DCs with a more anti-inflammatory profile [144]. Moreover, classical DCs are described as a source of IL-23 required for type 3 innate lymphoid cells (ILC3) activation, which are important producers of IL-22 and also by inhibiting commensal bacteria-specific $CD4^+$ T cell proliferation [145].

2.4.1.2. Innate lymphoid cells

Innate lymphoid cells (ILC) are a group of heterogeneous immune cells that are shown to be deeply involved in intestinal immune regulation and homeostasis [135]. ILC belong to the lymphoid lineage and are classified in three different populations according to their transcription factor expression and the production of signature cytokines: group 1, which include natural killer (NK) cells and type 1 ILC (ILC1) express T-box transcription factor Tbet and are IFN-γ and TNF-α producers; group 2 consists in ILC2, that express GATA3 and produce mainly IL-5 and IL-13-, and group 3, that comprises Lymphoid Tissue-inducer (LTi), which are important during fetal development as they are an essential for secondary lymphoid organ formation [146], and ILC3, expressing retinoic acid-related orphan receptor (ROR) γt and secrete IL-17A, IL-17F and IL-22 [147-149].

Due to the similarities in transcription factor expression and cytokine profile between ILC and $CD4^+$ Th1, Th2 and Th17, it has been suggested that ILC subsets may correspond to an innate counterpart of T cell populations [150]. Indeed, the arise of studies focused on ILC ontogeny has revealed that according with their developmental courses and function, a system with five subsets of ILC – NK cells, ILC1, ILC2, ILC3 and LTi cells – would be more accurate and therefore should be implemented [148].

Despite all ILC being involved in gut homeostasis, ILC3 embody a relevant role since they are highly responsive to extracellular microbial stimulation, such as bacteria and fungi [148]. This

ILC subset is the most abundant in mucosal tissues, representing the majority of ILC in the ileum and in the colon [151, 152]. The major function of ILC3 in the gut is to maintain epithelial barrier integrity in such a challenging environment [153]. ILC3 sense IL-23 and IL-1β in the gut and subsequently produce the effector cytokines IL-17, IL-22 and granulocyte-macrophage colony-stimulating factor (GM-CSF). They can also produce these cytokines after sensing microbiota through the aryl hydrocarbon receptor [154]. IL-22 presents a pivotal role in homeostatic control in the gut and is produced by Th17, Th22 and ILC3 cells. The latter are the main source of IL-22 in the intestine, placing themselves as crucial players in the protection and recovery of DSS-induced colitis and the related epithelial injury, since it is known that IL-22 is essential to maintain homeostasis by directly promoting epithelial cell protection, for instance, through the expression of antimicrobial peptides [155, 156]. ILC3-derived IL-22 can also control translocation of bacteria to the lamina propria. In fact, mice lacking ILC3 or IL-22 display impairments in the epithelial barrier and are unable to restrain commensal bacterial in the lumen. IL-22 secreted by ILC3 can be protective in mice against systemic colonization of pathogenic bacteria following *Clostridium difficile* infection and infection-induced injury of the epithelium [157]. ILC3 can also act as antigen-presenting cells due to the expression of MHCII. In fact, the deletion of MHCII in RORγt$^+$ ILC3 induces commensal bacteria-dependent intestinal inflammation, which suggests that ILC3 mediate intestinal CD4$^+$ T cell responses to commensal bacteria in a MHCII-dependent manner [158].

Research in IBD has pointed alterations in adaptive T cell responses as the main focus for the understanding of the immunopathology. Nonetheless, current findings have shown that ILC are capable to produce the same effector cytokines as CD4$^+$ and CD8$^+$ T lymphocytes, which indicates that despite being important players in the maintenance of intestinal homeostasis, these cell populations may also contribute to intestinal inflammation. Several associations were found between ILC dysfunction and IBD cases, mostly in CD. For instance, increased amounts of ILC2 have been detected in intestinal samples from CD patients, while the same was not observed in UC cases [159]. ILC3 can also contribute to intestinal inflammation through the production of GM-CSF, which promotes the recruitment of myeloid cells [160]. Anyway, a decreased frequency of ILC3 during commensal dysbiosis and, subsequently, lower amounts of IL-22 has been associated with the development of Th17-dependent colitis, which reinforces not only the importance of ILC3 in keeping gut homeostasis, but also the role of commensal microbes in suppressing pathogenic Th17 responses to commensal antigens [154].

2.4.2. Adaptive immunity

2.4.2.1. T cells

Intestinal T cells are present in a ratio of approximately 2/1 of $CD4^+$ T cells/$CD8^+$ T cells that derive from conventional T cells primed in secondary lymphoid organs [120]. $CD4^+$ T cells of the lamina propria have a miscellaneous repertoire, from $IL2^+$, IFN-γ^+ and $IL\text{-}17^+$ populations. IL-10-producing Treg cells, that express FOXP3, are also found in the gut. Along with Treg cells, the other subgroups of Th cells – Th1, Th2 and Th17 that, as it was previously described, share the same transcription factors of ILC1, ILC2 and ILC3, respectively – secrete specific cytokines and the regulation of these populations needs to be fine-tuned to maintain homeostasis [161-163]. The effector groups are essential to proper gut defense, such as pathogen infection or undue entry of luminal microbiota, but overactivation of these populations may generate intestinal inflammation and may be on the basis of IBD [164]. Th17 cells secrete IL-17, IL-22 and IFN-γ cytokines that when uncontrolled may lead to the development of an inflammatory response, due to the recruitment of neutrophils and upregulation of proinflammatory cytokines such as IL-1β, IL-6 and TNF-α, and eventually to the onset of colitis [165]. Therefore, IL-10-producing Treg cells control the proliferation of Th17 cells through RORγt inhibition by FOXP3. Besides, Treg cells can also inhibit the proliferation and function of Th1 and Th2 subsets through the secretion of IL-10 and TGF-β, which places them as major regulators of the inflammatory state of the intestinal mucosa [166].

Several reports state that CD and UC differ in terms of the adaptive immune response that is mounted during disease. Although CD and UC slightly differ in the cytokine profile, as CD seems to be mediated by IL-12, IFN-γ and TNF-α, while in UC there is also the contribution of IL-13, genome-wide association studies (GWAS) studies have confirmed that IL-23-driven Th17 responses have a key role in both UC and CD, with polymorphic variants associated to Th17 cell function being described in patients having one or other pathology [84, 167, 168].

2.4.2.2. B cells

B cells are mostly recognized in the inflammatory process by their ability to produce antibodies, despite being also able to release cytokines and act as secondary antigen-presenting cells [135]. The role of B cells in IBD is not as vastly explored as in the case of T cells, but they have also

been shown to contribute for the maintenance of intestinal homeostasis. B cells are able to produce immunoglobulin (Ig) A antibodies, which are capable to promote protection without eliciting an inflammatory response [169]. IgA is the main Ig isotype in the mucosal sites and thus act as the first immunological barrier in the body [170].

There are two subcategories of IgA in humans, IgA1 and IgA2, which vary in their distribution in the body. IgA1 is more predominant in the serum, while IgA2 have a more relevant role in mucosal secretions. Both IgA1 and IgA2 can also be presented as secretory IgA, a polymeric form which is known to provide protection of mucosal membranes by the neutralization of viruses or toxins and by its antibacterial activity [171]. The production of IgA is mostly dependent on the presence of the microbiota. Most plasma cells in duodenum and jejunum produce IgA1, while the prevalence of IgA2-producing cells increased from the small intestine to the colon [172]. IgA2 seems to be more adapted to bacteria-enriched environments and therefore is more abundant in the colon, since it was demonstrated that bacterial overgrowth in the small intestine can shift the IgA1 dominance to higher amounts of IgA2 [120].

The presence of circulating antimicrobial antibodies, such as anti-flagellin antibodies, where found in IBD patients but not in healthy controls, pinpointing that B cell reactivity occurs during disease [173]. For instance, anti-neutrophil cytoplasmic antibodies were found in sera from UC patients and fewer were also detected in CD patients [174]. Although not much is known about these antibodies and their impact in the disease, it has been shown that the number and higher titres of antibacterial antibodies is directly associated to a more severe clinical course, supporting that microbial antigens take part in the pathogenesis of IBD [175].

Overall, the development of IBD is associated with an imbalance of proinflammatory and immunosuppressive cells in the gut [135]. When microbial translocation occurs, immune cells are recruited, such as neutrophils, in order to perform microbial clearance. In parallel, macrophages, DCs and ILC act by recognizing antigens and producing cytokines to balance inflammation. Indeed, effector T cell functions can be suppressed by IL-10 produced by innate immune cells and also from Treg cells [127, 166]. The differentiation of Treg cells can actually be promoted by $CD103^+$ DCs [135, 141]. ILC, particularly ILC3, can act as antigen-presenting cells in a MHCII-dependent manner and restore epithelial barrier stability through the secretion of IL-22 [155, 156]. A strict balance between the vast immune repertoire present in the lamina propria, the epithelial barrier and the microbiota is thus needed to maintain intestinal

homeostasis and the understanding of the dynamic between effector and regulatory counterparts of the immune system is crucial to design better strategies for the management of IBD.

2.5. The crosstalk between gut microbiota, mucosal immunity and the epithelial barrier

The numerous microbes that inhabit the intestinal have co-evolved and established a complex symbiotic relationship with the host, in which the latter provides a stable environment enriched in nutrients perfectly adapted to act as microbial niche. On the other hand, the gut microbiota contributes by the digestion of complex dietary macronutrients, defense against pathogens, maintenance of the immune system and production of vitamins, metabolites and other nutrients that are otherwise unreachable to the host [176].

Intestinal microbes can impact human health by the production of beneficial metabolites that contribute to homeostasis and protection against disease. Dietary fibers and some proteins are metabolized in the cecum and colon by the microbiota. The major products derived from microbial fermentation are SCFAs, precisely acetate, propionate and butyrate [177]. Lactate is also a major organic acid resultant from fermentation of non-digestible carbohydrates [178], and it can also be subsequently metabolized to acetate, propionate and butyrate by several bacteria [179, 180].

It was observed that IBD patients evidence a decrease in SFCAs in the feces, such as acetate, lactate, butyrate and propionate, when compared to healthy controls, which supports the idea that SFCAs are crucial players in preventing the pathogenesis of IBD [181]. Recent advances in metagenomics have given the chance to characterize the bacteria responsible for SCFA production. Although acetate synthesis seems to be ubiquitously distributed among bacterial groups, the pathways for propionate, butyrate and lactate production seem highly conserved [182]. For instance, propionate appears to be produced by a rather few bacteria. Among those, *Akkermansia muciniphila*, a mucin-degrading bacterium, was identified as a major propionate and acetate producer [177, 183, 184]. A small number of microorganisms, such as *Faecalibacterium prausnitzii*, *Eubacterium rectale*, *Eubacterium hallii* and *Ruminococcus bromii*, seem to contribute for the largest amount of butyrate that is produced [185].

The biosynthesis of the major SCFAs can vary according to the bacteria that are involved in the process. Acetate can be produced mostly from pyruvate by bacteria either via acetyl-CoA or via the Wood-Ljungdahl pathway, in which acetate is synthesized by two separate branches

[186]. Propionate is produced from succinate conversion to methylmalonyl-CoA through the succinate pathway, or also from acrylate with lactate as a precursor by the acrylate pathway [187], or even from the propanediol pathway, in which deoxyhexose sugars serve as substrates [188]. Finally, butyrate can be produced by the condensation of two molecules of acetyl-CoA followed by reduction to butyryl-CoA, which can be converted to butyrate via the classical pathway [189]. Butyrate can also be synthesized by intestinal bacteria using lactate and acetate [177].

Several studies have shown how microbial populations and their derived metabolites can impact the intestinal environment. A recent study has demonstrated the crosstalk between SCFAs synthesized by microbiota and increased intestinal barrier function through the stabilization of epithelial hypoxia-inducible factor (HIF) [190]. Moreover, butyrate is one of the main metabolic substrates for epithelial energy homeostasis [191]. Studies investigating dysbiosis in IBD have found a positive association between decreased bacterial diversity, specifically lower abundance of butyrate-producing organisms such as *Faecalibacterium* and *Roseburia* genera, and the decreased amounts of butyrate present during inflammation [192]. A study focused on the composition of the ileal mucosa-associated microbiota has reported that a reduction on *F. prausnitzii* was associated with an increased risk of postoperative recurrence of ileal CD; it was stated that *F. prausnitzii* displayed an anti-inflammatory effect by the production of butyrate that was able to block NF-κB activation [193]. Others have described that butyrate regulates intestinal macrophage function by the inhibition of histone deacetylases and subsequent alteration of the expression of genes involved in cell proliferation and differentiation [194].

Acetate and propionate can also contribute to immune balance in the gut, since they can act as ligands to G-protein coupled receptor 43 (GPR43), which is expressed by Treg cells and therefore promote their expansion and immunosuppressive features such as IL-10 production, subsequently controlling proinflammatory responses [195]. Others have shown the beneficial role of propionate for intestinal homeostasis by the upregulation of the transcription factor Krüppel-like factor 4 (KLF4), which is associated with goblet cell differentiation and mucus formation and thus is important for intestinal epithelial homeostasis [196, 197].

2.6. Genetic associations in IBD

Genetic alterations are an important part of the puzzle when it comes to the understanding the mechanisms behind IBD pathology. Diverse GWAS have identified more than 200 alleles that are associated with IBD, 37 of which are specific for CD [198]. Nevertheless, there are already described several loci associated only with CD or UC, or with both disorders [2].

Alterations in *NOD2* gene are very well characterized, mostly in CD patients. This gene, as previously described, plays a key role in the detection of bacterial peptidoglycans and in the activation of cell signaling cascades. Therefore, *NOD2* is essential for bacterial recognition and to keep the balance between immune responses and commensal bacteria [199]. It is suggested that polymorphisms in *NOD2* may impair bacterial clearance and increase inflammation. The participation of *NOD2* in diverse events such as induction of autophagy and modulation of adaptive immune response complicates the understanding of which specific mechanism derived from *NOD2* function is involved in IBD [199-201]. GWAS have also pointed towards variations in *ATG16L1*, a component of autophagy complex, in CD, suggesting a functional integration of autophagy with microbial sensing and endoplasmic reticulum stress in this pathology. Since autophagy regulates several immune activities, namely innate and adaptive immune responses and processing of microorganisms, alterations in this gene may in part explain the onset of CD [202, 203]. Additionally, *IL10* is another gene whose impairment may lead to the development of UC. GWAS have shown that UC patients evidence polymorphisms in this gene, and concomitant results were found in mice lacking *IL10*, which spontaneously develop colitis [131, 132].

Alterations in other genes seem to be involved in the pathogenesis of IBD, such as in *MUC2*, *IL23R*, *STAT3*, *IRGM*, *SLC11A1*, among others [16, 204]. Nevertheless, genetic associations seem to account for only 20% of the genetic variance in susceptibility to IBD [2], failing to explain the divergent phenotypes that are found among patients. Thus, genetics are one of the partners in the dynamic of IBD pathology, composed also by immunity and microbiome.

3. EXPERIMENTAL MODELS OF IBD

3.1. Animal models of the disease

The advances in the understanding of IBD pathology have been vastly aided by the numerous animal models to study the disease. Although these models do not capture the entire complexity of the human pathology, they share several similarities that allow the retrieval of valid information regarding the mechanisms underlying the disease [205-207].

It is already well established that several factors intricately contribute to the development of IBD. There is a myriad of animal models for IBD research, mostly murine, that allow the study of these features, contributing for the current knowledge about mucosal immunity in IBD. Presently, there are chemically-induced models of IBD that accurately mimic the human pathology, as well as mice models with specific genetic or immunologic defects that can be at the genesis of the inflammatory process in IBD. The possibility of manipulate the intestinal microbiome in murine models have also helped to study the impact of specific microorganisms on intestinal inflammation.

3.1.1. Chemical models

3.1.1.1. Dextran sulfate sodium (DSS)-induced colitis

One of the most common models of IBD is the dextran sulfate sodium (DSS)-induced colitis. This model is used to mimic human UC and is based in the disruption of the intestinal epithelial barrier and subsequent entry of luminal bacteria or bacterial antigens to the lamina propria. DSS has a toxic effect to colonic epithelium, damaging epithelial cells and resulting in inflammatory immune response and impairment of barrier function [206]. Other authors have stated that the mode of action of DSS in colitis induction pass by the interaction with medium-chain fatty acids that reside in the lumen, forming nano-vesicles that fuse within the membrane and reach the cytoplasm, triggering intestinal inflammatory signaling pathways [208]. DSS colitis is considerably similar to human colitis and it is characterized by the formation of ulcers and erosion, loss of the crypts and infiltration of granulocytes.

Depending on the concentration (usually ranges between 1 to 5%) and the time of exposure, mice treated in the drinking water with DSS can develop acute colitis, chronic colitis or even colitis-induced dysplastic lesions [209]. This model of colitis can be established in the

absence of adaptive immunity, such as in Rag1-deficient mice and in severe combined immunodeficiency (SCID) mice, showing that the cytokines produced by innate cells are sufficient to induce inflammation. Thus, this is a valid research tool to unveil the mechanisms involving innate immunity and IBD, such as the importance of macrophages in the production of proinflammatory cytokines and cytokines that regulate epithelial barrier function, and the impact of neutrophils in intestinal tissue damage [210, 211]. Moreover, since the basis of DSS-induced colitis is the disruption of the epithelial barrier, this model places itself as a prominent model to unravel the dynamics underlying the maintenance and establishment of integrity during or after intestinal injury.

3.1.1.2. Trinitrobenzene Sulfonic Acid (TNBS)-induced colitis

This chemical model of IBD is based on the intrarectal administration of TNBS and it induces discrete foci of necrosis and inflammation that will trigger immune responses to self-antigens and thereby activates a mucosal immune response able to promote colitis in susceptible mouse strains [207]. The transmural colitis is mainly driven by Th1-mediated immune response, with inflammatory infiltration of the lamina propria by $CD4^+$ T cells, macrophages and neutrophils. TNBS-induced colitis shares similarities with CD, being therefore used as model to study the immune response in this disease, as well as potential therapeutic adjuncts [206].

3.1.2. Adoptive transfer colitis

The T cell transfer model to study chronic colitis has been essential to unveil the immune mechanisms that are behind the induction of the disease and also in the regulation of intestinal inflammation. The adoptive transfer of $CD4^+CD45RB^{high}$ T cells (naïve T cells) from healthy wild-type mice to recipient mice lacking both T and B cells leads to the development of a severe generalized colitis, accompanied with small intestine inflammation at five to eight weeks following T cell transfer. This procedure leads to transmural inflammation, epithelial cell hyperplasia, epithelial cell erosion and leukocyte infiltration, and weight loss and loose stool whose degree varies according to the mouse strain [212, 213].

Since both colon and small intestine develop inflammation upon T cell transfer, it makes this a model close to CD. This model is also very relevant to study the impact of Treg cells in the suppression the onset of intestinal inflammation, allowing the study the early events associated with the development of the disease and its subsequent evolution [214].

3.1.3. Genetic models

A plethora of genetically engineered animals is available to study IBD. Several of them were identified to spontaneously develop colitis/ileitis, playing a key role in the study of the basic mechanisms of IBD development and also possible therapeutic interventions.

3.1.3.1. IL-10-deficient mice

IL-10 knockout (ko) mice are genetically manipulated for the absence of *IL10* gene and are known to spontaneously develop colitis after approximately 12 weeks of age. The microbiota composition also impacts the susceptibility of IL-10 ko mice to develop the disease, since the presence of *Helicobacter* species seem to be detrimental for the animals. These mice develop chronic transmural enterocolitis, mucosal hyperplasia and a severe inflammatory response [215]. It is reported that this colitis model seems to be mediated mainly by $CD4^+$ T cells and dependent on the proinflammatory effect of IL-17, IL-6 and IL-23 [207].

3.1.3.2. NOD2-deficient mice

Mice deficient in *NOD2* gene are also commonly used in the study of IBD pathophysiology. Defects in *NOD2* gene were already described in IBD patients and therefore animal models shaping these alterations are a great advantage to study possible pathways involved in IBD [216, 217]. NOD2-deficient mice are known to be more susceptible to colitis (although they do not develop spontaneously the disease) either induced by DSS or TNBS, evidencing also an increased risk for azoxymethane (AOM)/DSS-induced colorectal cancer [218, 219]. Besides, NOD2-ko mice are also used in a model of infection with *Salmonella enterica* serotype Typhimurium that creates a CD-like symptoms, such as intestinal fibrosis, which allows the study of the genetic factors involved in intestinal fibrosis and the impact of *NOD2* in the susceptibility on CD [220].

3.1.3.3. MUC2-deficient mice

Having a model that specifically targets the intestinal epithelium and mucus layer is also a groundbreaking tool to deeper understand the dynamics of IBD. MUC2-deficient mice are animals that do not express *MUC2*, the gene encoding for mucin 2. This mucin is localized in the apical granules of the goblet cells and is the major responsible for mucus production in the

intestine [94]. These mice are more susceptible to DSS-induced colitis and may also develop spontaneous colitis during life [94, 221]. The histological architecture of the gut in MUC2-ko mice is altered and it is believed that it is the lack of a proper physical barrier that enables commensal bacteria to reach the lamina propria and induce inflammation [94, 221, 222]. This is a valuable model to study IBD since it provides a new insight regarding the impact of impairment of the intestinal epithelial barrier in the onset of IBD.

3.2. Alternative models: gut-on-a-chip platforms

The use of *in vitro* models can be useful to screen putative candidate factors or dissect possible mechanisms underlying certain pathologies. Nevertheless, the currently available *in vitro* models lack on physiological relevance. The conceptualization of *in vitro* organ-on-a-chip systems aimed to overcome the limitations found in conventional *in vitro* models by mimicking the basic functioning of *in vivo* tissue environment [223, 224]. The combination of *in vivo* models with these new technologically evolved *in vitro* systems provides a groundbreaking research strategy for an integrative analysis, covering the phenotype observed *in vivo* as well as the cellular mechanisms unveiled *in vitro*.

Specifically focused on the intestine, several research groups have developed gut-on-a-chip systems that replicate the three-dimensional physiological properties and functions of the intestine, such as mucus production and villi formation [225]. These microfluidic devices are usually composed by small chambers connected by microchannels that support fluid flow. With the help of a peristaltic pump, it is possible to perfused the system with culture medium at desired flow rates, allowing to recapitulate the fluid flow and shear stress of cell surface that are found in the human intestinal lumen. The majority of the intestine chips has a porous extracellular (ECM)-coated polycarbonate membrane separating the two compartments of the chip, in which immortalized human intestinal epithelial cells (Caco-2 or HT-29 cell lines) are seeded. The monolayer that is formed is normally used to assess several parameters regarding epithelial barrier stability, such as tight junction barrier function and the absorption of compounds, as drugs or nutrients [225, 226].

The interaction of epithelial cells and microbiota counterparts were also taken into account in the development of this technology. Recently, it was described a system that separates a luminal microbial compartment from the epithelium by a nanoporous membrane, aiming to aid the survival of a host-microbiome environment comprising both epithelial cells and anaerobic gut

bacteria, by a constant perfusion of culture medium [227]. Notwithstanding, the absence of mechanical deformations simulating peristaltic movements may be detrimental for microbial growth [228]. Moreover, the physical separation promoted by the membrane to these two compartments, as well as the fact that these co-cultures are only able to survive for approximately 24 hours, limits its application for long-term characterization of host-microbial interactions.

Gut-on-a-chip models are much more reliable to characterize phenotypically and functionally the human intestine than conventional *in vitro* cultures, although there are still some gaps that need to be explored. For instance, the inclusion of nervous system cells may be the next step since it is known that enteric nervous system has a role in the regulation of intestinal secretion and gut motility [229]. Therefore, and despite being in a very inceptive stage, these systems provide increased robustness over conventional *in vitro* cultures and with further advances may be an innovative tool to explore intestinal dynamics and validation of personalized medical approaches.

4. CONTEXT AND AIMS OF THE THESIS

IBD is a chronic debilitating disease with an increasing incidence around the world. One of the major setbacks in the clinics regarding IBD treatment is the multifactorial profile of this pathology, which dampens the efficacy of currently available therapies. Therefore, it is paramount to fully understand the mechanisms that are involved in IBD and which factors can be modulated to promote protection. Despite the etiology of IBD is not entirely determined, it is known that dynamic interaction between the gut microbiota, the immune system and the intestinal epithelial barrier is pivotal for the maintenance of a healthy intestine and that disruption of this balanced ecosystem can lead to the development of IBD.

Aim 1: To identify the colonic microbiota and metabolic signatures associated with a healthy intestinal environment.

Aim 2: To define the colonic epithelial barrier components and the immune landscape targeted by specific microbiota populations responsible for promoting protection against colitis.

Aim 3: To characterize the metabolic profile in the intestine during colitis development and identify altered nutrients that can be targeted for dietary supplementation to potentially contribute to disease resolution.

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CHAPTER II

AKKERMANSIA AND *PARABACTEROIDES* SPECIES ENRICHMENT AMONG THE COLONIC MICROBIOTA ASSOCIATES WITH PROTECTION AGAINST COLITIS

Akkermansia and *Parabacteroides* species enrichment among the colonic microbiota associates with protection against colitis

IN BRIEF

Colonic enrichment in *Akkermansia muciniphila* and *Parabacteroides distasonis* in the intestine is associated with protection against ulcerative colitis development. The protective effect is related with a beneficial effect on gut epithelial barrier that becomes more prepared to sustain an inflammatory insult.

HIGHLIGHTS

- Genetically similar mice from different animal facilities evidenced a striking opposite phenotype upon chemically induced colitis, with a group of mice displaying a remarkable protection against disease development.

- The protective phenotype is associated with an overexpression of genes encoding for proteins associated with intestinal epithelial barrier function, such as claudins and mucins as well as higher levels of IL-10, IL-17 and IL-22.

- Resistant mice also display increased number of goblet cells per crypt under homeostatic conditions when compared to susceptible mice, reinforcing the presence of a healthier epithelial barrier.

- The resistance phenotype is transmissible by fecal microbiota transplant of stool contents from resistant to susceptible mice.

- A combination of metagenomic and metabolomic analyses allowed to identify microbial organisms and metabolic products associated with the protective phenotype.

- Among the species found to be statistically enriched in resistant mice, *A. muciniphila* and *P. distasonis* were found to be the most significantly increased in mice displaying a natural or fecal microbiota transplant-induced resistant phenotype.

ABSTRACT

Inflammatory bowel disease (IBD) is a group of inflammatory conditions of the gastrointestinal tract. Crohn's disease (CD) and ulcerative colitis (UC) are the principal forms of the disease. The etiology of IBD remains elusive, but it is known that this pathology arises from the interaction of environmental and genetic factors that trigger inadequate immune responses and inflammation in the intestine, which in turn affect the balance in gut microbiota and the intestinal epithelial barrier. While chemically inducing colitis in mice from two distinct animal facilities, we unexpectedly observed that one group showed remarkable resistant to disease development. A combination of metagenomic and metabolomic analyses demonstrated that the two groups of mice have distinct microbiota and metabolic signatures. We identified *Akkermansia muciniphila* and *Parabacteroides distasonis* as significantly increased in the microbiota of resistant mice. Indeed, these bacteria can be at the genesis of the protective phenotype, since by fecal microbiota transplant (FMT) from resistant to susceptible mice, the latter become not only protected against colitis, but also enriched in these bacteria. Resistant mice have increased levels of IL-10, IL-17 and IL-22 and also higher expression of claudin- and mucin-encoding genes in homeostatic conditions as compared to susceptible mice. Simultaneously, by testing the bacterial candidates in colonic epithelial cells using a gut-on-a-chip platform, we found increased expression of E-cadherin when cells were incubated with *A. muciniphila*, which is in accordance with the previous *in vivo* findings. These results suggest that protection may be mediated by an intestinal epithelial barrier more prone to sustain an inflammatory insult. We are currently exploring the mechanisms by which these bacteria impact intestinal epithelial cells to induce protection against inflammation, using the gut-on-a-chip platform to then validate these findings in an *in vivo* susceptible model of colitis. These findings may pave the way towards the identification of novel players to promote a healthy intestinal environment, as well as promising therapeutic targets for IBD treatment.

INTRODUCTION

The mammalian gastrointestinal tract hosts a vast community of microbes, namely bacteria, fungi and viruses. These microorganisms have coevolved and established mutualistic relationships with the host, contributing for the complex ecosystem that is found in the intestine [1, 2]. This nutrient-rich microbial niche provided by the host is in turn compensated by the microbial contribution through the generation of vitamins, metabolites and other nutrients that are not accessible for the host unless via microbial metabolism [3]. A myriad of pathogen recognition receptors, such as Toll-like and NOD-like receptors, mediate the interaction between the cells present in the intestinal epithelial barrier and the microbiota by sensing and responding to the microbiota, which contributes to the maintenance of intestinal homeostasis through the secretion of factors such as antimicrobial peptides and mucus [4-6]. Also, the presence of an intestinal epithelial barrier avoids an excessive contact between microbial populations and the immune system [7]. A fine-tuned equilibrium between the microbiota, immune system and barrier function is therefore essential for a proper intestinal function and to maintain homeostasis. Nevertheless, disruptions in one or more of these counterparts may lead to functional imbalances and subsequently trigger intestinal pathologies, such as inflammatory bowel disease (IBD) [8].

IBD is a multifaceted inflammatory disorder with a considerable high disease burden in the world [9]. Comprising both Crohn's disease (CD) and ulcerative colitis (UC), IBD has been challenging the medical intervention approaches due to the lack of information regarding the events leading to its inherent pathology and subsequently hampering the development of efficient therapies [10-12]. Although the etiology of IBD is still elusive, impairments in the intricate interaction between the gut microbiota composition and immune recognition, often enhanced by genetic susceptibility factors, have been pinpointed as the main cause for the occurrence of this disease [10, 13].

While analyzing dextran sulfate sodium (DSS)-induced colitis in genetically similar C57BL/6 mice derived from two distinct animal facilities, we serendipitously observed a group of animals with a remarkable resistance to disease development. This protective phenotype was associated with an increased expression of claudin- and mucin-encoding genes in homeostatic conditions, as well as higher IL-10, IL-17 and IL-22 secretion. Moreover, we found that resistant and susceptible mice displayed a distinct metabolic profile, with 12 of the 37 identified

metabolites being significantly increased in resistant mice. Since a vast majority of the metabolites present in the gut are derived from bacterial metabolism, we analyzed the microbiota composition of these two groups of mice by metagenomics. We found that susceptible and resistant mice have a distinctive microbiota composition and, particularly, we identified *Akkermansia muciniphila* and *Parabacteroides distasonis* as significantly enriched in resistant mice. These bacteria can be at the genesis of the protective phenotype, since fecal microbiota transplant (FMT) from resistant to susceptible mice was able to reverse colitis susceptibility. The putative protective effect of these bacterial candidates will be evaluated in an *in vivo* model of colitis that is currently ongoing. Moreover, the mechanism by which these bacteria can modulate intestinal function, namely epithelial barrier integrity, will be assessed in an optimized *in vitro* gut-on-a-chip platform. Using this intestinal environment mimicking device, we obtained preliminary results suggesting the beneficial effect of *A. muciniphila* on epithelial barrier, with increased expression of E-cadherin by epithelial cells. Hence, the findings collected in this work have shown that *A. muciniphila* and *P. distasonis* can act as beneficial players in promoting protection against ulcerative colitis, presumably by enhancing intestinal epithelial barrier and better sustaining an inflammatory insult. By pinpointing the mechanisms underlying the protective action of these bacteria, this work may give new insights towards a better understanding of IBD immunopathology.

MATERIAL AND METHODS

Animals

C57BL/6 mice used in this study were derived from two different animal facilities. While one group were purchased from Charles River Laboratories (France), the other group was previously originated from the same commercial enterprise, but was housed and bred at ICVS Animal Facilities, under specific pathogen-free (SPF) conditions, for more than 15 generations. All animals used for FMT or colonization with specific bacteria were housed in biosafety level 2 facilities. Mice were euthanized by CO_2 inhalation with efforts to minimize suffering. All experimental procedures were performed in accordance with the relevant guidelines and regulations. The animal studies were approved by the Ethical Council for Life and Health Sciences at the University of Minho (SECVS 003/2018), using FELASA guidelines and recommendations for laboratory animal experimentation.

Colitis induction

Mice with 7 to 9 weeks old were administered with dextran sulfate sodium (DSS; 3% (w/v), molecular weight approximately 40000 Da; TdB Consultancy) in the drinking water *ad libitum* for 7 days. Clinical signs of colitis were monitored daily and measured by the disease activity index (DAI). DAI comprises weight loss, stool consistency and bleeding assessment and it is obtained by the sum of the scores of each parameter (Table 2.1). Mice were euthanized in the end of each experiment or earlier, if the symptoms of clinical disease reached one of these endpoints: more than 20% weight loss (relatively to the initial weight), diarrhea or gross bleeding. For chronic colitis model, 2% DSS was given in the drinking water for 5 days in two subsequent phases with an interval of 3 weeks.

Table II.1. Disease activity index (DAI) scores.

Score	Weight loss	Stool consistency	Bleeding
0	No loss	Normal	No blood
1	1-5%	Mild-soft	Brown color
2	6-10%	Very soft	Reddish color
3	11-20%	Diarrhea	Bloody stool
4	> 20%		Gross bleeding

The final score is obtained by the sum of each parameter.

Histological evaluation

Samples from colons were fixed in 4% paraformaldehyde and 5 µm paraffin-embedded sections were stained with hematoxylin and eosin. Inflammation was assessed in a blinded fashion by a pathologist, using a semi quantitatively-graded system as previously described (Table 2.2; [14]). Staining with Alcian Blue/Periodic Acid-Schiff (AB/PAS) of colon section was performed to evaluate polysaccharide structures. Goblet cell number was assessed for each experimental condition in a blinded fashion. Only intact crypts, cut longitudinally from crypt opening to bottom, were quantified. Images were captured using an Olympus BX61 microscope and recorded with a digital camera (DP70) using Cell^P software. Image analysis was performed using Fiji (ImageJ) software.

Table II.2. Parameters for histological analysis of colitis severity.

Score	Epithelial hyperplasia and goblet depletion	Leukocyte infiltration in the Lamina Propria	Affected area	Markers of severe inflammation
0	None	None/rare	None	None
1	Minimal	Increased	1/3	Increased
2	Mild	Confluent	2/3	Confluent
3	Marked	Transmural	All	Transmural

The final score is obtained by the sum of individual scores. Markers of severe inflammation included ulceration and crypt abscesses.

FITC-dextran intestinal permeability assay

In vivo intestinal permeability was assessed using a fluorescein isothiocyanate (FITC)-labelled dextran administration. Food and water were withdrawn for 8 hours. Mice were administered with 44 mg/100 g body weight of FITC-labelled dextran (TdB Consultancy; 4 kDa) by oral gavage. Serum was collected four hours later and fluorescence intensity was measured by spectrophotofluorimetry (excitation: 485 nm; emission: 528 nm). Serum was diluted 1:2 in PBS and concentrations were determined by correlation with a linear standard curve made using diluted FITC-labelled dextran in serum from untreated mice.

RNA extraction, cDNA and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from colonic samples using TripleXtractor (Grisp) with mechanical disruption of the tissues on ice, followed by convertion into cDNA by reverse transcription with Xpert cDNA synthesis kit (Grisp). qRT-PCR was performed using KAPA SYBR FAST Universal

(Roche) on a Bio-Rad CFX6 Real-Time System C1000 Thermal Cycler (Bio-Rad). Specific oligonucleotides for mouse mucin-encoding genes *Muc1*, *Muc2*, *Muc4* and *Muc13*, for claudin-encoding genes *Cldn2*, *Cldn3*, *Cldn4*, *Cldn7* and *Cldn8*, for *Cdh1* (E-cadherin) and *Ocld* (occludin) are shown in Table 2.3. Assays were performed using the following protocol: one cycle of 95°C for 3 minutes, followed by 40 cycles of a two-stage temperature profile of 95°C for 3 seconds and 60°C for 30 seconds. Expression levels were normalized to ubiquitin (*Ubq*) and relative expression was determined based on the ΔCt method, as follows: $2^{(\text{housekeeping gene mRNA expression - Target gene mRNA expression})} \times 100000$ [15].

Table II.3. List of primers used for PCR.

Primer ID	Forward sequence (5'→ 3')	Reverse sequence (5'→ 3')
Cdh1	CACCTGGAGAGAGGCCATGT	TGGGAAACATGAGCAGCTCT
Cldn2	GGCTGTTAGGCACATCCAT	TGGCACCAACATAGGAACTC
Cldn3	AAGCCGAATGGACAAAGAA	CTGGCAAGTAGCTGCAGTG
Cldn4	CGCTACTCTTGCCATTACG	ACTCAGCACACCATGACTTG
Cldn7	AGGGTCTGCTCTGGTCCTT	GTACGCAGCTTTGCTTTCA
Cldn8	GCCGGAATCATCTTCTTCAT	CATCCACCAGTGGGTTGTAG
Muc1	CCCTATGAGGAGGTTTCGGC	AAGGGCATGAACAGCCTACC
Muc2	TCCTGACCAAGAGCGAACAC	ACAGCACGACAGTCTTCAGG
Muc4	AGGACCATCGTGCTCTCTCT	AGCATACTTAGGTTCAGAGCCA
Muc13	CTGGCAGCTACATGAGCACT	GAACTACCCACGGTCACCAA
Ocld	GCTGTGATGTGTGTTGAGCT	GACGGTCTACCTGGAGGAAC
Ubq	TGGCTATTAATTATTCGGTCTGCAT	GCAAGTGGCTAGAGTGCAGAGTAA
Am	CAGCACGTGAAGGTGGGGAC	CCTTGCGGTTGGCTTCAGAT
Pd	TGCCTATCAGAGGGGGATAAC	GCAAATATTCCCATGCGGGAT

Lamina propria leukocyte (LPL) isolation and flow cytometry analysis

To isolate lamina propria leukocytes (LPL), colons were flushed with Ca- and Mg-free PBS with 25 mM HEPES (Gibco), 50 mM sodium bicarbonate (Sigma-Aldrich) and 5% fetal bovine serum (FBS; Gibco). Then, colons were opened longitudinally and cut into 0.5 to 1 cm pieces. These fragments were incubated in Ca- and Mg-free Hank's Balanced Salt Solution (HBSS; Gibco) containing 1.3 mM EDTA (Sigma-Aldrich), 25 mM HEPES, 50 μg/mL penicillin/streptomycin (Gibco) and 2 mM L-Glutamine (Gibco), under 200 rpm agitation at 37°C for 40 minutes. Fragments were then transferred to new flasks and were incubated in RPMI 1640 medium

(Gibco) supplemented with 0.15 mg/mL collagenase D (Roche), 10% FBS, 25 mM HEPES, 50 μg/mL penicillin/streptomycin and 2 mM L-Glutamine for 40 minutes under 200 rpm agitation at 37°C, before dissociation of the tissue and filtration through a 70 μm cell strainer (BD Biosciences). Cell suspension was centrifuged and the pellet was resuspended in 40% Percoll (GE Healthcare), laid over 80% Percoll and centrifuged at 600g for 20 minutes at 20°C. Cells retained in the interface were collected, washed in RPMI containing 2% FBS and recovered. Cells were enumerated in 4% trypan blue on a hemocytometer.

Surface staining was performed with antibodies specific for mouse CD45 (Brilliant Violet (BV) 510; clone 30-F11), CD90.2 (Thy1.2) (Pacific Blue; clone 53-1.2), CD3 (PE; clone 145-2C11), CD4 (APC-Cy7; clone GK1.5), CD19 (BV650; clone 6D5), CD11c (BV605; clone N418), CD11b (PE-Cy7; clone M1/70), Ly6C (PerCP-Cy5.5; clone HK1.4); Ly6G (BV711; clone 1A8) and MHCII (FITC; clone M5/114.15.2) (Biolegend). All staining was performed for 30 minutes at 4°C. Cells were fixed before acquisition with 1% paraformaldehyde in PBS for 30 minutes. For intracellular staining, cells were stimulated with 50 ng/mL phorbol myristate acetate (PMA) and 500 ng/mL ionomycin calcium salt for 3.5 hours, followed by the addition of 10 μg/mL brefeldin A for 1.5 hours, at 37°C. All stimuli were purchased from Sigma-Aldrich. Cells were collected after stimulation and then surface stained with the antibodies described above, followed by intracellular staining for RORγt (APC; clone B2D) and IL-17A (FITC; clone TC11-18H10.1), using the BD Permwash Kit (BD Biosciences) as per manufacturer's instructions. Cell analysis was performed on a BD LSRII (Becton Dickinson, USA). Data were analyzed using FlowJo software (Tree Star, USA).

Cytokine quantification by ELISA

Colonic tissues were weighted and lysed using a homogenizer in ice-cold PBS containing protease inhibitors (Roche). Protein concentrations were quantified using the Pierce BCA protein assay kit (Bio-Rad). The levels of IL-10, IL-17A/F and IL-22 were measured by ELISA using commercially available kits (Biolegend), according to manufacturer's instructions.

Metabolomic analysis by Nuclear Magnetic Resonance (NMR)

Frozen colons were homogenized and colon metabolites were extracted with a methanol:chloroform protocol for frozen samples. Aqueous fraction from colon samples and sera were analyzed on a Bruker Avance III 800 MHz spectrometer equipped with a 5 mm TXI-Z H

C/N/-D probe with gradients. One dimensional 1H-NMR spectra was obtained using the first transient of noesy-presaturation pulse sequence and with using a CPMG pulse sequence with water presaturation. Two dimensional NMR experiments, including homonuclear (1H-1H) total correlation spectroscopy and heteronuclear (1H-13C) single quantum spectroscopy, were performed to aid spectral assignments. These were further confirmed through database search (Human Metabolome Database) and resorting to ChenomxNMRSuite software. Data were analyzed by different multivariate analysis using SIMCA software. Relevant metabolite differences identified were further tested by univariate analysis of the corresponding spectral intensities using line-deconvolution algorithms.

Microbiota modulation

Co-housing. C57BL/6 mice from two different animal facilities were co-housed at 4 weeks old at the same cage, for a period of 4 weeks. Fecal samples were collected prior and at the end of the co-housing for bacterial 16S rRNA gene sequencing.

Antibiotic treatment for microbiota depletion. C57BL/6 mice were given ampicillin (1 mg/mL), streptomycin (1 mg/mL), vancomycin (0.5 mg/mL) and neomycin sulfate (1 mg/mL) in the drinking water for 4 weeks. All antibiotics were purchased from Sigma-Aldrich. Microbiota depletion was assessed throughout the treatment by aerobic and anaerobic culture of intestinal contents in Columbia agar plates with 5% sheep blood at 37°C. The number of colony-forming units (CFU) was counted and the number of bacteria per mg of feces calculated. As a control for the depletion procedure, fecal pellets from mice prior antibiotic treatment were collected and subjected to the above described protocol.

Fecal microbiota transplant (FMT). Fresh fecal contents from resistant mice were directly collected to a sterile 2 mL capped microtube, resuspended in ice-cold phosphate-buffered saline (PBS; Gibco) and centrifuged (800g for 5 minutes) to remove residual clumps. A 150 μL/day of the resuspended material was given by oral gavage to mice from the susceptible group. For the experiments of FMT following antibiotic treatment, the procedure was performed during 3 days, while for the chronic model, where antibiotic treatment was not performed, FMT was performed for 5 days during disease remission (between cycles of DSS treatment). The mice that did not

receive FMT were administered with drinking water by oral gavage during the same period of time, in order to match the stress of the procedure.

Metagenomic analysis and bacterial quantification

Genomic DNA from feces was extracted using the QIAamp Fast DNA Stool Mini Kit (Qiagen) according to manufacturer's instructions plus an additional membrane disruption step using glass beads. After quantification of genomic DNA by spectrophotometry at 260 nm, 16S rRNA gene was amplified and sequenced using the MiSeq platform from Illumina and analyzed with mothur as previously described [16]. Sequences were aligned to the 16S rRNA gene upon filtering by quality and length, using as a template the SILVA reference alignment program. Operational taxonomical units (OTUs) were identified by the average-neighbour algorithm. Sequences with distance-based similarity of 97% or higher were grouped into the same OTU. Each sequence was classified using the Bayesian classifier algorithm with a bootstrap cutoff of 60%. Two-tailed Wilcoxon non-parametric test was applied to identify significant microbiota taxonomic changes among groups. The false discovery rate (FDR) approach was used to adjust for multiple hypothesis testing. Changes with a $p < 0.05$ and FDR < 0.2 were considered significant.

Absolute abundance of bacteria was performed by quantification of bacterial copy number in stool DNA samples using specific primers for *Akkermansia muciniphila* (Am) and *Parabacteroides distasonis* (Pd) (Table 3). Values were extrapolated from a standard curve obtained by different copy numbers of a plasmid containing a specific insert belonging to each bacterium. For the plasmid generation, a specific DNA fragment for *A. muciniphila* or *P. distasonis* was inserted in a pJET1.2 plasmid using a CloneJET PCR Cloning Kit (Thermo Scientific). After transfection in *E. coli*, plasmid DNA was obtained and was used as template for the standard curve.

Bacteria growth

Akkermansia muciniphila (DSM-26127) and *Parabacteroides distasonis* (DSM-29491) were purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Leibniz-Institut, Germany). *A. muciniphila* was grown in brain heart infusion (BHI) medium (Thermo Scientific) supplemented with 0.1% of porcine mucin (Sigma-Aldrich) and *P. distasonis* was grown in Wilkins-Chalgren broth (Thermo Scientific), in a chamber with an anaerobic

atmosphere composed by 80% N_2, 10% O_2 and 10% CO_2, at 37°C. Columbia agar plates with 5% sheep blood were also used to for solid cultures of both bacteria.

Gut-on-a-chip model

Biochips. Biochips were made from polystyrol and obtained from Microfluidic ChipShop GmbH (Jena, Germany), and manufactured as described in [17]. Biochip upper and lower chambers have 700 μm and 400 μm of height, respectively. Afferent and efferent channels have a width of 0.8 mm and 2 mm. The volumes of each chamber are 220 μL for the upper chamber and 120 μL for the lower, and the cavities are separated by a 12 μm polyethylene terephthalate (PET) membrane with a pore diameter of 8 μm.

Cell culture. Caco-2 cells (epithelial cell line from colorectal adenocarcinoma) were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% FBS (Gibco), and 100 U/mL penicillin plus 100 μg/mL streptomycin mixture (Thermo Fisher). Human umbilical vein endothelial cells (HUVECs) were isolated and cultured as previously described [18, 19]. Primary blood mononuclear cells were isolated from healthy donors by Ficoll density gradient centrifugation and seeded in 6-well plates with a density of 1 x 10^6 cells/cm^2 in X-VIVO 15 (Lonza) supplemented with 10% autologous serum, 100 U/mL penicillin plus 100 μg/mL streptomycin mixture (Thermo Fisher), 10 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) and 10 ng/mL macrophage colony-stimulating factor (M-CSF). GM-CSF and M-CSF were added to induce and enhance macrophage polarization.

Biochip assembly for bacteria testing. To mimic the epithelial cell layer, Caco-2 cells were cultured and seeded at a density of 2.5x10^6/mL in the upper cavity of the chip and placed in an incubator at 37°C with 5% CO_2. Briefly, in the day after Caco-2 seeding, the chip was connected to a circuit with peristaltic pumps and medium perfusion was started with a rate of 50 μL/min. After 7 days, Caco-2 cells presented a tridimensional conformation similar to the villi present in the gut. Since we want to test two anaerobic bacteria strains, oxygen concentration inside the incubator was decreased gradually, as follows: day 8 with 10% O_2, day 9 with 5% O_2, and day 10 with 1% O_2. Total absence of oxygen was not assessed in these experiments since previous data showed that Caco-2 cells do not tolerate well the absence of oxygen from longer periods. Then, at day 11, bacteria suspensions (*A. muciniphila*, *P. distasonis*, or the combination of both) at an

OD_{600} = 0.2 were added on top of the epithelial cells, with a perfusion rate adjusted to 15 µL/min. Epithelial cells without the addition of bacteria were tested as control. After 24 hours, cells were fixed and stained for E-cadherin.

Biochip assembly for bacterial supernatant testing. HUVECs were seeded at a density of $2.5x10^6$/mL in the lower cavity of the chip and placed in an incubator at 37°C with 5% CO_2. After two days, monocyte-derived macrophages were seeded at a density of $1x10^6$/mL on top of HUVECs. The seeding of Caco-2 cells and the perfusion was performed as previously described. At day 10, half of the media circulating in the upper chamber (epithelial side) was replaced by bacteria culture supernatants from *A. muciniphila*, *P. distasonis*, or the combination of both. This experiment was performed under normoxic conditions. Supernatants were collected at 24 and 48 hours for cytokine quantification and stored at -80°C.

Immunofluorescence. Membranes were extracted from the biochip and cells were fixed with 10% paraformaldehyde for 10 min at room temperature. Subsequently, cells were permeabilized and unspecific binding sites blocked with PBS including 0.1% saponin and 3% goat serum. Staining was done with E-cadherin (Life Technologies) antibody and goat anti-mouse-Cy3 (Invitrogen), with 4',6-diamidino-2-phenylindole (DAPI) counterstaining. Samples were embedded in fluorescent mounting medium (Dako) and imaged with an AxioObserver Z1 fluorescence microscope equipped with an ApoTome-2 (Carl Zeiss AG, Jena, Germany).

Statistical analysis

Metabolite differences were evaluated by ANOVA in R statistical software. Partial least squares – discriminant analysis (PLS-DA) models were performed in SIMCA software. For multiple group comparisons t-test or one-way ANOVA test with a Tukey multiple-comparison posttest were performed, while for multiple group comparisons with repeated measures two-way ANOVA test with a Tukey multiple-comparison posttest was applied. Images are representative of at least 3 independent experiments. Data are presented as mean ± standard deviation (SD). Statistically significant values are: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

RESULTS

Mice from different animal facilities display different susceptibility to chemically-induced colitis

In mammals, the immune system and metabolic homeostasis are vastly intertwined and impact each other by numerous mechanisms. For instance, during inflammatory responses several biological functions, such as metabolic processes, are adjusted to restore homeostasis [20]. The immune and metabolic systems have co-evolved so they can sense alterations in the environment and thus respond to new conditions in order to maintain host's homeostasis [21].

To unveil how immunometabolic interactions account for IBD development, we chemically induced colitis in wild-type C57BL/6 mice by DSS treatment for seven days. All the parameters associated to disease progression, such as weight loss, stool consistency and the presence of blood in the stool were monitored daily and scored according to the DAI. Surprisingly, we observed that wild-type C57BL/6 mice did not display major clinical signs of disease development even when exposed to higher concentrations of DSS, or prolonged treatment (Figure II.1A-B). Simultaneously, colitis was induced in a similar protocol to wild-type C57BL/6 mice housed in a different animal facility. As expected, these mice developed colitis, presenting an average DAI score of 8 (out of 10) at day 7 post DSS administration (Figure II.1B). Upon examination, susceptible mice had short, thick colons, consistent with significant colon pathology (Figure II.1C). Histologic analysis of the colons, comprising ulceration, structural alterations (crypt shortening or ablation) and the presence of inflammatory infiltrates, also showed a severe histopathology in the susceptible mice when compared to those without severe disease progression (Figure II.1D-E). Interestingly, the total number of goblet cells is also dissimilar between susceptible and resistant mice already at homeostatic conditions. Consistently, a massive abrogation of goblet cells and the mucus layer was observed only on susceptible mice upon DSS-induced colitis (Figure II.1F-G). Hence, despite being genetically identical and subjected to same experimental protocol, mice from different animal facilities evidenced a different response upon colitis induction, with one group developing colitis (susceptible group), whereas in the other group there was an absence of disease progression (for a matter of simplicity, this group of mice will be referred as resistant throughout this thesis).

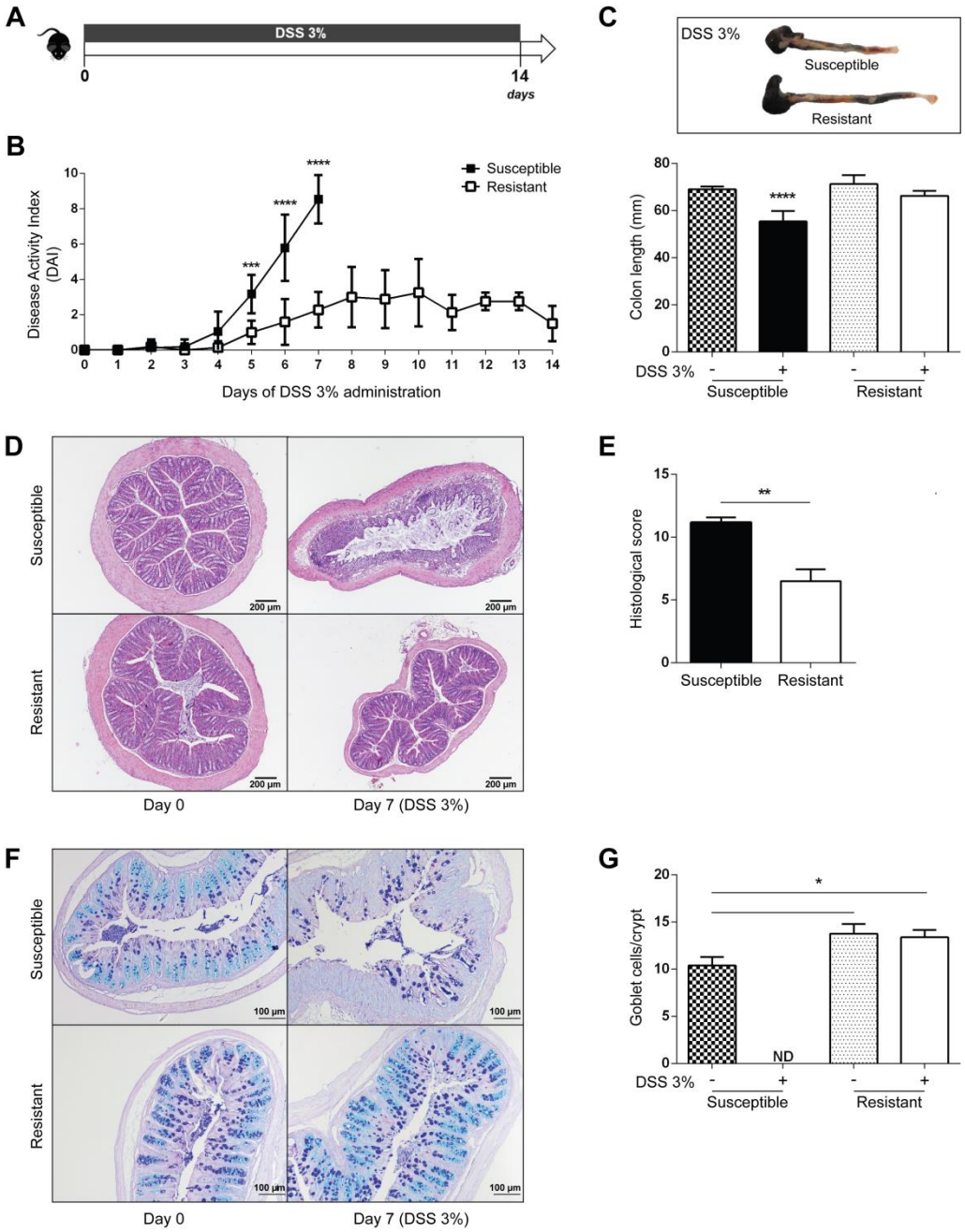


Figure II.1. Mice from different animal facilities display distinct susceptibility to colitis development. **(A)** C57BL/6 mice from two different animal houses were administered with dextran sulfate sodium (DSS) 3% in the drinking water and were monitored daily. **(B)** Disease progression was assessed by scoring the disease activity index (DAI) throughout the experiment. **(C)** Representative colons were imaged and colon length was measured at day 7, after excision. **(D)** Histological analysis of hematoxylin & eosin staining of mice prior and after colitis induction. **(E)** Colitis scores were obtained by the histological evaluation of colon samples at day 7. **(F)** Alcian blue/periodic acid-Schiff staining of the colonic tissues for goblet cells and mucus analysis. **(G)** Quantification of goblet cell numbers per crypt. For susceptible mice at day 7, no intact crypts were found; ND – not detected. Images are representative of at least three independent experiments; n=8/10 per group. Data is presented as mean ± standard deviation (SD). Statistically significant values are: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$.

Resistant mice display an upregulation of genes associated with epithelial barrier function

Since the two groups of mice have the same genetic background but a divergent phenotype upon colitis induction, we hypothesized that alterations in the stability and function of the intestinal epithelial barrier could be associated with the observed phenotype. Impairments in the intestinal epithelial barrier, such as exacerbated intestinal permeability, are known to be a hallmark in IBD and are found in IBD patients [22]. Yet, no significant differences were found in the intestinal permeability among the two groups of mice in homeostatic conditions (i.e., prior colitis induction) that could be associated with the distinct outcome of disease development (Figure II.2A).

Besides impairments in intestinal permeability, alterations in the expression of tight junctions and adherens junction proteins were described in IBD patients, reinforcing the relevance of the integrity of the intestinal epithelial barrier in this pathology [23]. Although no major differences were found in the intestinal permeability among the two groups of mice, the transcriptional levels of mucin-encoding genes (*Muc1*, *Muc2* and *Muc13*) as well as the claudin and E-cadherin genes (*Cldn2*, *Cldn3*, *Cldn4*, *Cldn7* and *Cdh1)* were significantly upregulated in resistant mice when compared to the susceptible group (Figure II.2B-C). Of note, no significant differences were found in the expression of *Muc4*, *Cldn8* and *Ocln*, which encode for mucin-4, claudin-8 and occludin, respectively (Figure II.2C). The expression of proteins encoded by these genes is undoubtedly intertwined with epithelial barrier stability, which is known to be paramount in controlling intestinal inflammatory conditions. Mucins, particularly mucin-2, are important proteins involved in the synthesis and maintenance of the mucus layer. Indeed, impairments in the mucus layer are associated with severe colitis and therefore it can promote the development of colitis [24]. Similarly, alterations in the levels of claudins and cadherins, responsible to maintain paracellular permeability and tight junction integrity and function, are described to be found in patients with intestinal inflammatory conditions. Particularly, impaired expression of claudin-3, -5 and -8 and occludin were described in patients with Crohn's disease [23], as well as down-regulation of claudin-1 and -4 and increased expression of claudin-2 in ulcerative colitis patients [25]. However, these data obtained on homeostatic conditions suggests a hyperactivation of epithelial barrier associated proteins, which may prepare the epithelial barrier to sustain an inflammatory insult. As such, upon colitis induction the susceptible group presented increased levels of dextran in the serum when compared to resistant mice (Figure II.2D)

mirroring the differences in colitis score (Figure II.1B) and corroborating our previous hypothesis that resistant mice may present a more prepared epithelial barrier to hold inflammation.

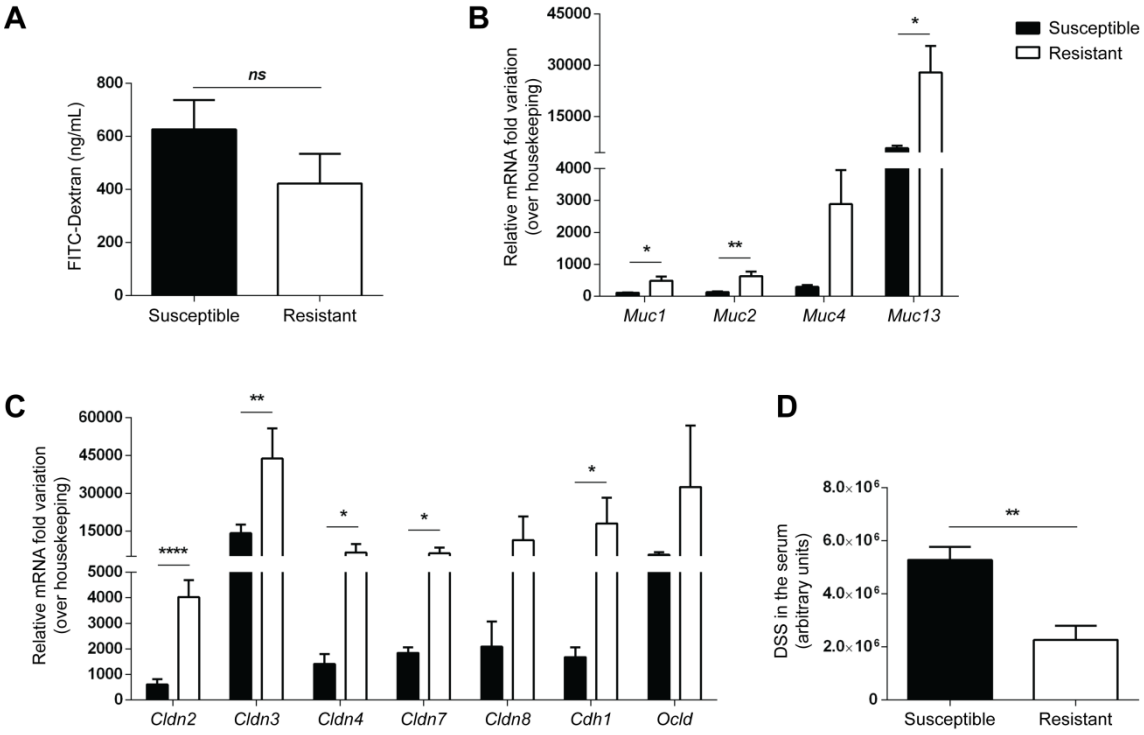


Figure II.2. Resistant mice have increased expression of genes involved in epithelial barrier function. **(A)** Intestinal permeability in homeostasis was measured after administration of FITC-Dextran by oral gavage and quantified in the serum after four hours of administration. **(B-C)** Expression of *Muc1*, *Muc2*, *Muc4*, *Muc13* (B), *Cldn2*, *Cldn3*, *Cldn4*, *Cldn7*, *Cldn8*, *Cdh1* and *Ocld* (C) was analyzed by qPCR in homeostatic conditions. **(D)** Permeability was assessed after colitis induction (day 7 of DSS administration) by the quantification of dextran in the serum by NMR. Images are representative of at least three independent experiments; n=8/10 per group. Data is presented as mean ± standard deviation (SD). Statistically significant values are: $*p < 0.05$; $**p < 0.01$; $****p < 0.0001$. ns – not significant.

Protective phenotype is associated with increased frequency of type 3 innate lymphoid cells and cytokine production

To determine whether the protective phenotype observed in resistant mice was influenced by a different pattern of immune cells in the gut prior colitis induction, the distinct immune cell populations were evaluated by flow cytometry (Figure II.3A). Most adult tissue macrophages are originated during embryonic development and not from circulating monocytes [26]. Yet, it is established that body's barrier tissues, such as skin, lungs and intestine, are continuously replenished by blood Ly6C^{hi} monocytes [27]. Full monocyte maturation, as well as the acquisition of a characteristic functional signature by gut mucosa macrophages, is essential for

the maintenance of intestinal homeostasis. However, this process is impaired during inflammatory events, when infiltrating Ly6C^{hi} monocytes respond to microenvironmental factors and commensal microbiota, becoming pro-inflammatory. This is particularly relevant since the disruption of the differentiation pattern of monocytes in this tissue plays a critical role in IBD [28]. We found that resistant mice have decreased frequency of myeloid cells, namely neutrophils, Ly6C^{hi} monocytes and Ly6C^{+}MHCII^{+} monocytes (Figure II.3B). The frequency of macrophages in the gut was similar between the two groups of mice. B cells similarly have an increased frequency in susceptible mice when compared to resistant phenotype (Figure II.3B).

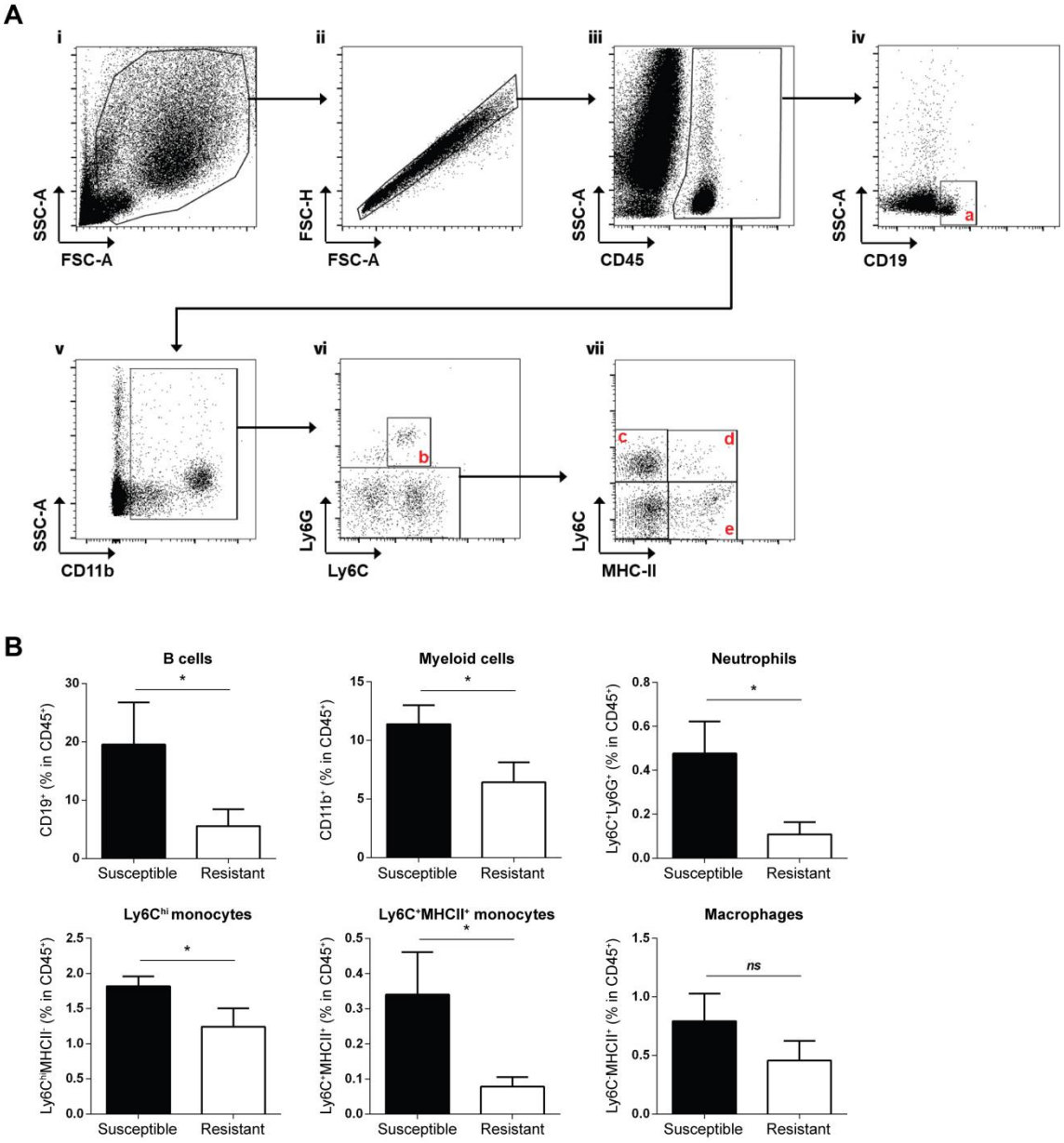


Figure II.3. Resistant mice present decreased frequency of myeloid and B cell populations in homeostatic conditions. (A) Representative plots showing the gating strategy used for the identification of myeloid cell populations and B cells within lamina propria leukocytes. Cells were selected by forward scatter (FSC)

and side scatter (SSC) profile (i) and excluding doublets (ii). Hematopoietic cells were defined by positive expression of CD45 (iii). B cells were obtained by gating cells expressing CD19 (iv; a). Within myeloid cells (positive for CD11b; v), neutrophils (vi; b), Ly6C^{hi} monocytes (vii; c), Ly6C^{+}MHCII^{+} monocytes (vii; d) and macrophages (vii; e) were assessed. **(B)** Frequencies of B cells and myeloid populations present in the gut of susceptible or resistant mice, under homeostatic conditions. Images are representative of at least three independent experiments; n=5/8 per group. Data is presented as mean ± standard deviation (SD). Statistically significant values are: $*p < 0.05$. ns – not significant.

To further characterize the immune environment in the gut of both susceptible and resistant mice prior colitis induction, the cytokine profile was evaluated. Significant differences were found for IL-10, IL-17 and IL-22 production, which are increased in resistant mice (Figure II.4A). IL-10 is known to play a key role in intestinal homeostasis [29] and thus is concordant with the protective phenotype that was identified for these mice. IL-17 is a pro-inflammatory cytokine that is associated with several autoimmune and inflammatory pathologies and IL-22 is a cytokine belonging to IL-10 family of cytokines that can have both proinflammatory and tissue-protective properties depending on the context in which it is expressed [30, 31]. Both IL-17 and IL-22 can be produced by a myriad of immune cells present in the gut, namely T helper 17 (Th17) cells and type 3 innate lymphoid cells (ILC3). We evaluated these cell populations in the gut to understand if they could be playing a role in the protective phenotype observed in resistant mice (Figure II.4B). We found that susceptible and resistant mice have a divergent frequency of both ILC3 and Th17 cells in the gut at homeostatic conditions, with resistant mice displaying a significantly higher frequency of ILC3 and, inversely, lower frequency of Th17 cells, when compared to susceptible mice (Figure II.4C). Interestingly, IL-17-producing ILC3 in resistant mice are decreased in comparison with susceptible mice. ILC3 are one of the major producers of IL-22 [32], and therefore this may suggest that ILC3 in resistant mice is producing other type of cytokines, such as IL-22 (Figure II.4C), which remain to be tested. Moreover, we also observed that although resistant mice display higher levels of IL-17 in the colon when compared to the susceptible group, they also present a decreased frequency of IL-17-producing cells. This can indicate that other cell populations may be contributing for the IL-17 that is found in the gut of resistant mice.

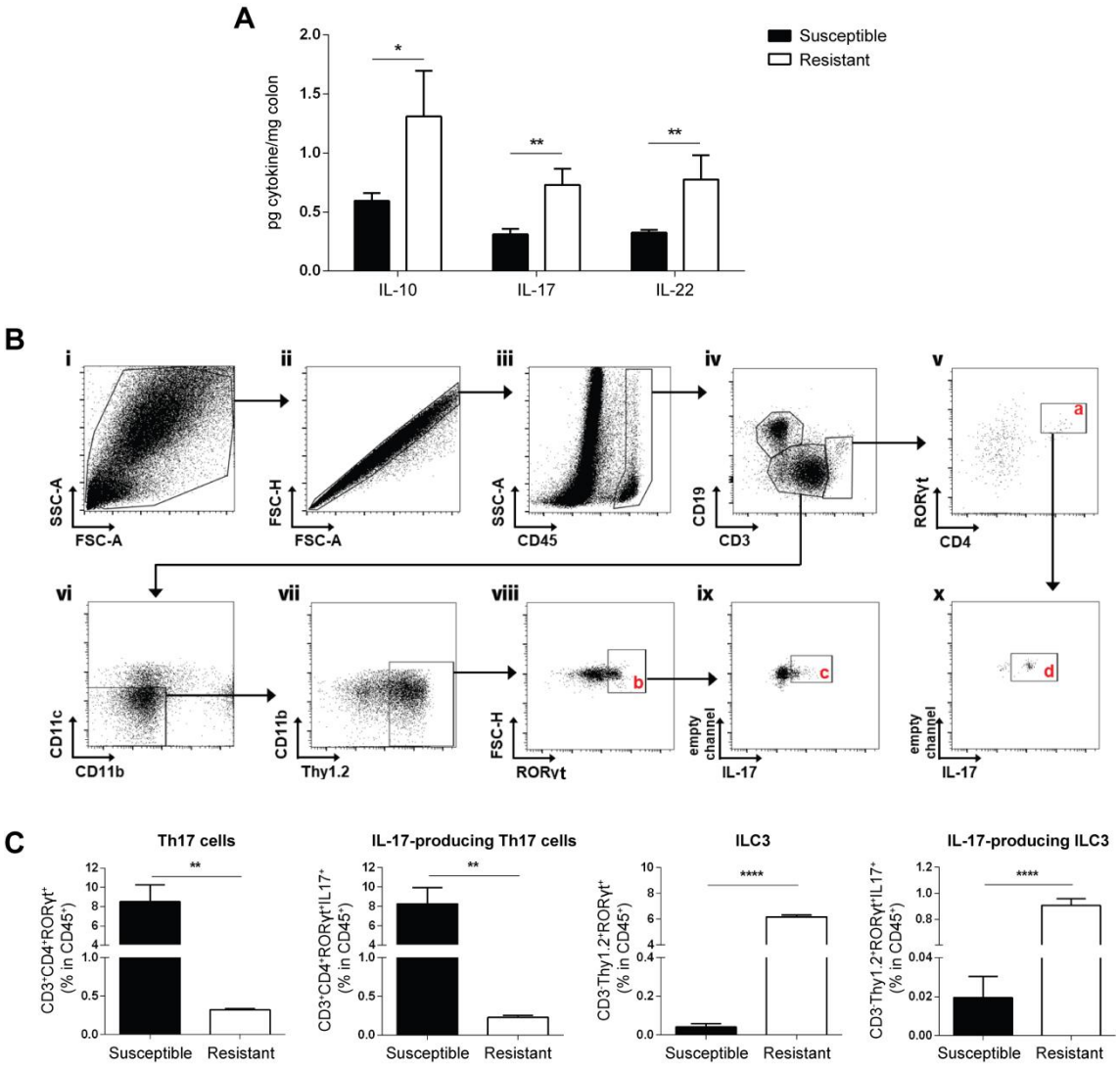


Figure II.4. Susceptible and resistant mice have a distinct cytokine profile and dynamic of ILC3/Th17 cells in the gut during homeostasis. (A) The production of IL-10, IL-17 and IL-22 (pg cytokine/mg colon) was quantified in colonic extracts at homeostatic conditions. **(B)** Representative plots showing gating strategy used for the identification of Th17 cells and ILC3. Cells were selected by forward scatter (FSC) and side scatter (SSC) profile (i) and excluding doublets (ii). After gating hematopoietic cells by CD45 expression (iii), cells were distinguished by the expression of CD3 (iv). Th17 cells were identified as $CD3^{+}CD4^{+}ROR\gamma t^{+}$ cells (v; a). ILC3 were identified as $CD45^{+}CD3^{-}CD19^{-}CD11b^{-}CD11c^{-}Thy1.2^{+}ROR\gamma t^{+}$ (viii; b). IL-17-producing cells within ILC3 and Th17 were identified in (ix; c) and (x; d), respectively. **(C)** Frequencies of Th17 cells, IL-17-producing Th17 cells, ILC3 and IL-17-producing ILC3 in the gut of susceptible or resistant mice, under homeostatic conditions. Images are representative of at least three independent experiments; n=5/7 per group. Data is presented as mean ± standard deviation (SD). Statistically significant values are: $*p < 0.05$; $**p < 0.01$; $****p < 0.0001$.

Microbiome is modulating protection against colitis development

Intestinal epithelial barrier integrity and immune cell populations can influence and also be shaped by the microbiome composition in the gut [33]. To determine if the protective phenotype upon colitis induction was driven by a distinctive microbiota composition, susceptible and resistant mice were co-housed during four weeks to share the same environment and subsequently the microbiome, followed by colitis induction (Figure II.5A). The co-housing protocol abolished the protective phenotype displayed by the resistant mice, which became equally susceptible after co-housing with mice from the susceptible group, displaying similar DAI scores and disease progression (Figure II.5B). This result demonstrates that, not only the microbiome is at the basis of the protective phenotype but also the microbiota responsible for the protective phenotype display a recessive pattern and have a decreased fitness when competing with the microbiota present in susceptible mice.

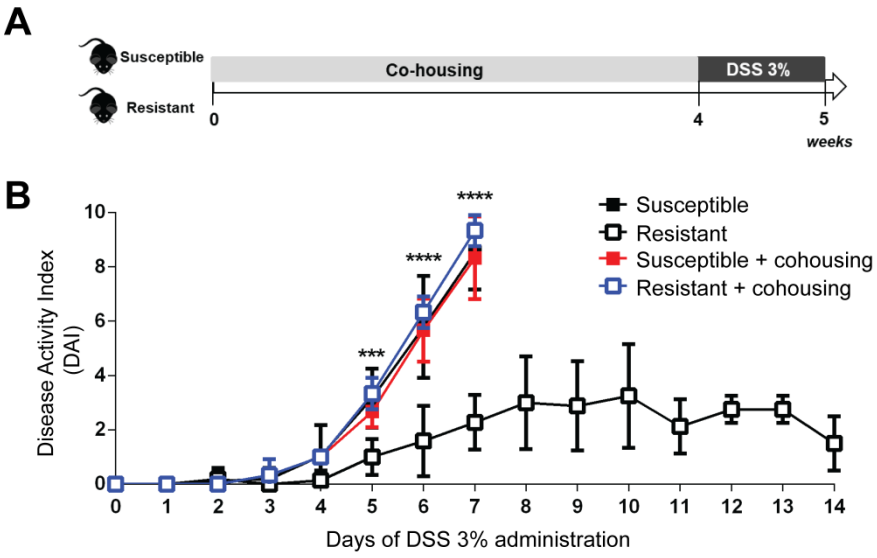


Figure II.5. The resistant phenotype lost in co-housing experiments points for a recessive nature of protective microbiome. (A) Four week-old susceptible and resistant mice were co-housed during 4 weeks and then were administered with DSS 3% for 7 days. **(B)** Disease progression was assessed by scoring the disease activity index (DAI) throughout the experiment. Images are representative of at least three independent experiments; n=5/10 per group. Data is presented as mean ± standard deviation (SD). Statistically significant values are: ***$p < 0.001$; ****$p < 0.0001$.

To address the question whether the microbiome was in fact underlying the protective phenotype found in one of the groups of mice, a FMT with fecal contents from resistant mice was performed into antibiotic-induced microbiota depleted susceptible mice (Figure II.6A). Basically,

susceptible mice were firstly treated with a mixture of antibiotics composed by a broad-spectrum antibiotic (ampicillin) with the narrow-spectrum antibiotics vancomycin that acts on Gram-positive bacteria, streptomycin active on Gram-negative bacteria and neomycin sulfate effective against Gram-negative and some Gram-positive bacteria. The efficacy of the microbiota depletion was assessed throughout the antibiotic treatment. Very little aerobic bacteria colony-forming units (CFUs) and undetected anaerobic bacteria CFU were assessed just upon five weeks of treatment (Figure II.6B). Hence, we established a five-week antibiotic treatment in susceptible mice prior to the FMT.

Antibiotic-induced microbiota depleted susceptible mice underwent a FMT protocol, in which each mouse orally received a fecal suspension from resistant mice, for three consecutive days (Figure II.6A). Following three weeks upon FMT colonization, mice were submitted to DSS treatment for colitis induction. A clear protection against colitis induction was observed in this group mirroring the resistant group as reflected by their similar DAI (Figure II.6C), which clearly proves that microbiome is the key player in the protective phenotype.

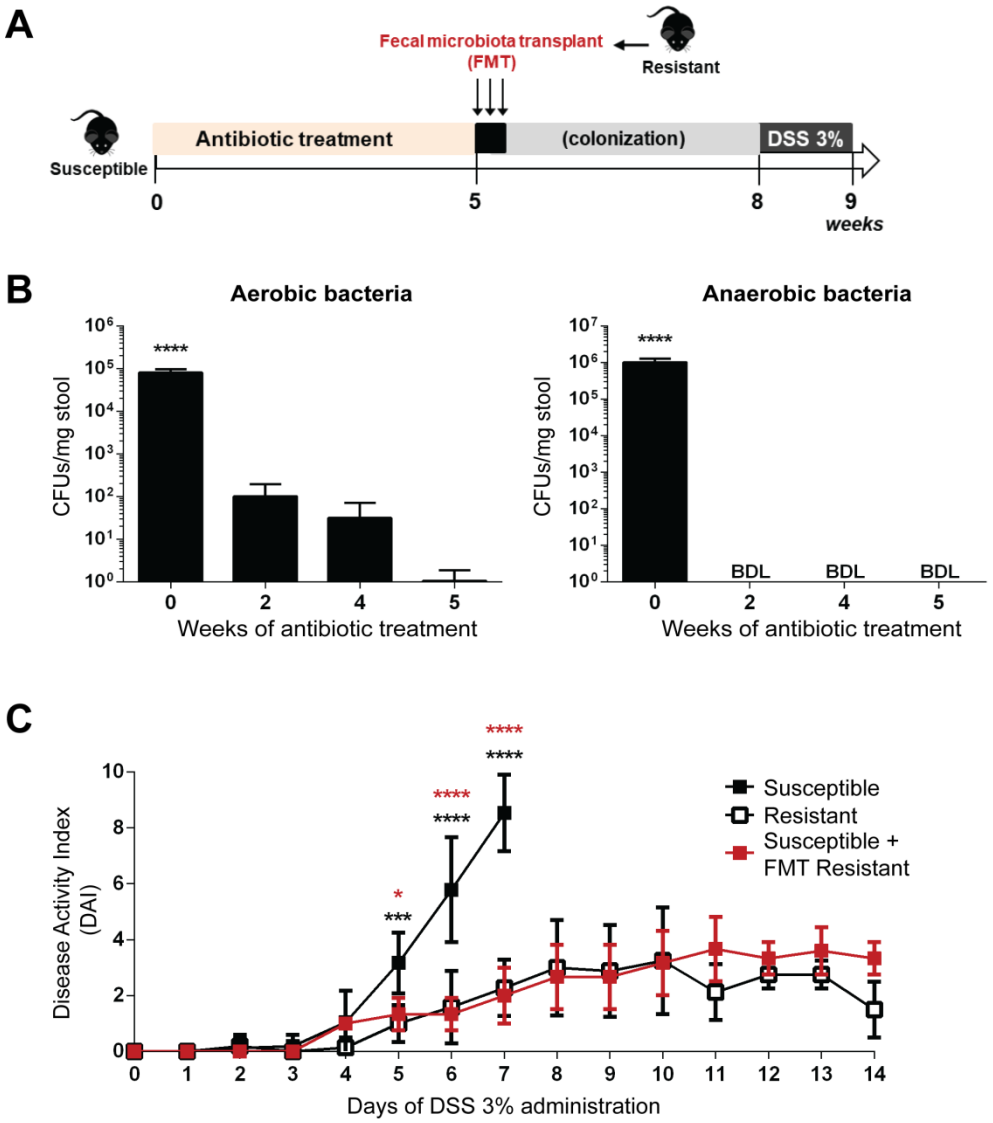


Figure II.6. Fecal microbiota transplant (FMT) from resistant to susceptible mice is sufficient to confer protection against colitis induction. (A) Susceptible mice were treated with antibiotic for 5 weeks and then received fecal contents from resistant mice by oral gavage during 3 days. After 3 weeks to allow colonization, mice were challenged with DSS 3% for 7 days. **(B)** Rate of microbiota depletion during antibiotic treatment was assessed by quantification of aerobic and anaerobic bacteria colony-forming units (CFU) in stool. **(C)** Disease progression was assessed by scoring the disease activity index (DAI) throughout the experiment. Images are representative of at least three independent experiments; n=5/10 per group. Data is presented as mean ± standard deviation (SD). Statistically significant values are: $*p < 0.05$; $***p < 0.001$; $****p < 0.0001$. BDL – below detection level.

This hypothesis was also corroborated in a relapse-remission model of colitis. Colitis was induced in susceptible mice and upon remission of the disease mice received FMT from the resistant mice by oral gavage during five days. In this experimental setting, no antibiotic treatment was administered. Two weeks after the FMT, mice were again challenged with colitis

(Figure II.7A). Mice treated with fecal contents from resistant mice displayed only mild symptoms of colitis, contrary to control group that only received the vehicle (PBS) (Figure II.7B). Although both groups evidence inflammation features in the histopathological analysis (both have developed colitis in beginning of the treatment and prior FMT), mice that received FMT displayed significantly fewer signs of pathology than the control group. Moreover, mice that received FMT also evidenced higher amount of goblet cells per crypt (Figure II.7C-D). This protection was even more striking by the fact that there was no previous microbiota depletion in these mice, which showed that simply by the enrichment with the microbiota from the resistant mice it was possible to induce protection in mice that were previously susceptible.

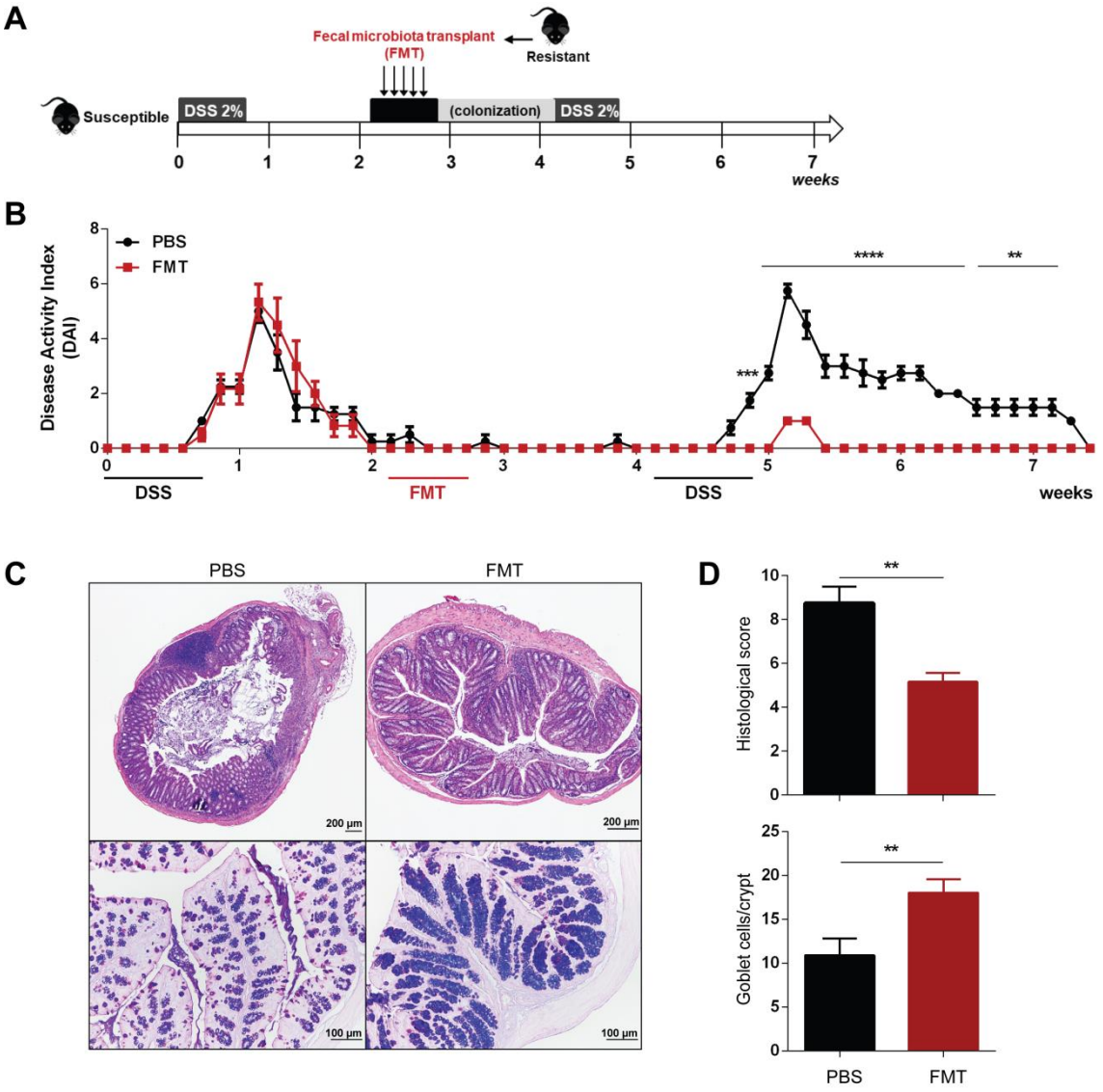


Figure II.7. Fecal microbiota transplant (FMT) from resistant to susceptible mice is able to avoid relapse in chronic colitis. (A) Susceptible mice were treated with DSS 2% for five days. After remission, mice received FMT from resistant mice by oral gavage for 5 days. Control group was treated with the vehicle (PBS). Two

weeks later, both groups were given DSS 2% as previously. **(B)** Disease progression was assessed by scoring the disease activity index (DAI) throughout the experiment. **(C)** Histological analysis of hematoxylin & eosin and Alcian blue/periodic acid-Schiff stainings of the colonic tissues from mice that received FMT or PBS at 7 weeks of treatment. **(D)** Colitis scores were obtained by the histological evaluation of colon samples at week 7. Quantification of goblet cell numbers per crypt. Images are representative of at least three independent experiments; n=5 per group. Data is presented as mean ± standard deviation (SD). Statistically significant values are: $^{**}p < 0.01$, $^{***}p < 0.001$; $^{****}p < 0.0001$.

Metagenomic analysis revealed a distinct gut microbiota composition between susceptible and resistant mice

Our results clearly demonstrate a crucial role of gut microbiota in conferring protection against colitis. To further investigate this hypothesis, we performed a metagenomic analysis of gut microbiota composition by analyzing the stool of mice, both susceptible and resistant, in homeostatic conditions. This approach allows the identification of several bacterial genera that are present in both groups of mice and therefore to retrieve those that can be at the basis of the protective phenotype.

The metagenomic analysis provided a qualitative distinction between susceptible and resistant mice according to the presence or absence of certain bacteria and their relative abundance. A representative heat map summarizes some relevant identified bacterial genera between both groups (Figure II.8A). Susceptible and resistant mice clustered separately in an unsupervised multivariate analysis of the microbiota composition indicating a considerable difference among the microbial populations (Figure II.8B). The graphical representation of the relative abundance of the 129 identified bacterial genera reinforces the different microbiota composition between resistant and susceptible mice (Figure II.8C). Quite unexpectedly, the resistant group of mice present a significantly reduction of richness, measured by the Sobs index that quantifies the number of operational taxonomic units or number of species (OTUs, similar to the phylogenetic level of species; Figure II.8D) and species diversity measured by the Shannon index (Figure II.8E).

We also extended our analysis to susceptible mice that received FMT from resistant ones, since they displayed a protective phenotype after FMT and therefore it can contribute to the identification of the bacteria associated with protection. Interestingly, this group of mice also diverges from the others displaying an intermediate phenotype between susceptible and resistant regarding the gut bacterial composition (Figure II.8B). Interestingly, no significant differences

were observed on the susceptible group before or after the FMT in terms of the relative abundance of bacterial genera, number and diversity of species (Figure II.8C-E) Although these results could be seen as somewhat surprising given the similar protective phenotype against colitis induction displayed by the susceptible + FMT and resistant groups, this may also suggest that microbial minority populations could be responsible for the protective phenotype. Overall, by identifying what is different between resistant and susceptible mice, and then what is present in resistant mice that is enriched in susceptible after FMT, we can pinpoint the bacterial candidates that can be at the genesis of protection against colitis.

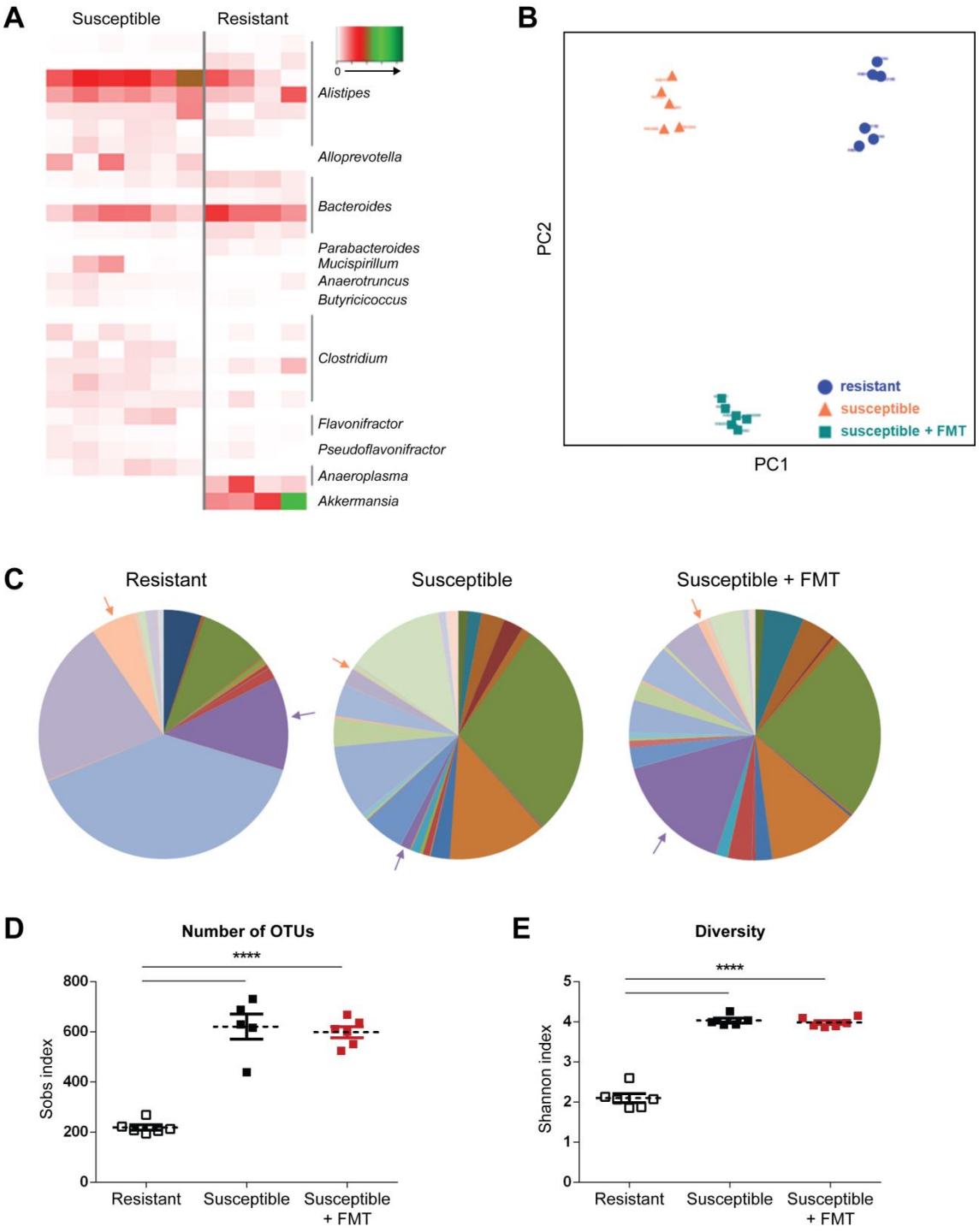


Figure II.8. Metagenomic characterization of resistant and susceptible mice revealed a distinct microbiota composition (A) Representative heat map of genera identified from DNA from stool samples of susceptible and resistant mice. **(B)** Principal coordinate analysis (PCoA) based on bacterial community similarity of susceptible, resistant and susceptible + FMT mice. **(C)** Relative abundance of bacterial genera; *A. muciniphila* and *P. distasonis* are marked by purple and beige arrows, respectively. **(D)** Number of operational taxonomic units (OTUs) and **(E)** diversity of species found in susceptible, resistant and susceptible + FMT mice. Images are representative of at least three independent experiments; n=5/6 per group. Data is presented as mean ± standard deviation (SD). Statistically significant values are ****$p < 0.0001$.

Akkermansia and *Parabacteroides* species are significantly increased in the gut microbiota community of mice protected against colitis development

To identify the most promising bacterial candidates associated with protection, we analyzed thoroughly the metagenomic data obtained with the three previous groups. A multivariate analysis identified several bacteria genera to be significantly increased in resistant mice when compared to the susceptible group (Figure II.9A). Among these, we found that *Akkermansia*, *Parabacteroides*, *Lactobaccilus*, *Ureaplasma* and *Bacteroides* genera were the most significant hits as represented by the adjusted *p* values. Intersecting this with the metagenomic data of susceptible mice after FMT, allowed to pinpoint *Akkermansia* and *Parabacteroides* as the most significantly increased when a resistant phenotype was observed. Since these two genera encompass different species, 16S sequences generated by the MiSeq platform from Illumina for each OTU identified as *Akkermansia* or *Parabacteroides* were used in a Basic Local Alignment Search Tool (BLAST) analysis to confirm the identification at a species level. We found that the most representative species for both genera were *Akkermansia muciniphila* and *Parabacteroides distasonis*. Therefore, these candidates were selected to evaluate their putative role in the protection against colitis.

To confirm that *A. muciniphila* and *P. distasonis* were in fact increased in mice that showed protection against colitis induction, i.e. resistant mice and also susceptible mice that received FMT, we examined the absolute abundance of these bacteria. For that, we used specific primers for 16S rRNA sequences of both bacteria and assessed the number of copies in DNA extracted from stool samples of mice. As expected, *A. muciniphila* and *P. distasonis* were significantly increased in both resistant and susceptible mice after FMT, when compared to susceptible mice (Figure II.9B). In fact, the abundance of *A. muciniphila* and *P. distasonis* in resistant mice is, on average, 500000 and 15000 times higher, respectively, than in susceptible mice, while for susceptible mice after FMT the abundance of *A. muciniphila* and *P. distasonis* is 3000000 and 7000 times higher than before receiving FMT. Overall, this result confirms the significant representativeness of these two species in the gut microbiota of mice that are able to be protected against colitis, pointing towards a possible effect of these bacteria, alone or in combination, in creating a very particular niche that allows intestine to sustain an inflammatory insult such as colitis induction.

A

Increased in resistant mice (*vs* susceptible)			Increased in susceptible + FMT mice (*vs* susceptible)		
Genus	***p* value**	**Adjusted *p* value**	**Genus**	***p* value**	**Adjusted *p* value**
Akkermansia	0.00131	0.01132	*Akkermansia*	2.25×10^{-9}	0.00153
Parabacteroides	0.00205	0.01284	*Parabacteroides*	0.00028	0.00860
Lactococcus	0.00560	0.02426	*Bacteroides*	0.00085	0.01447
Ureaplasma	0.00756	0.03067	*Olsenella*	0.00342	0.02909
Bacteroides	0.00835	0.03203	*Coprococcus*	0.00928	0.04856
Parasutterella	0.04786	0.10961	*Anaeroplasma*	0.01691	0.06764
			Ureaplasma	0.02011	0.07199
			Turicibacter	0.03900	0.12056

B

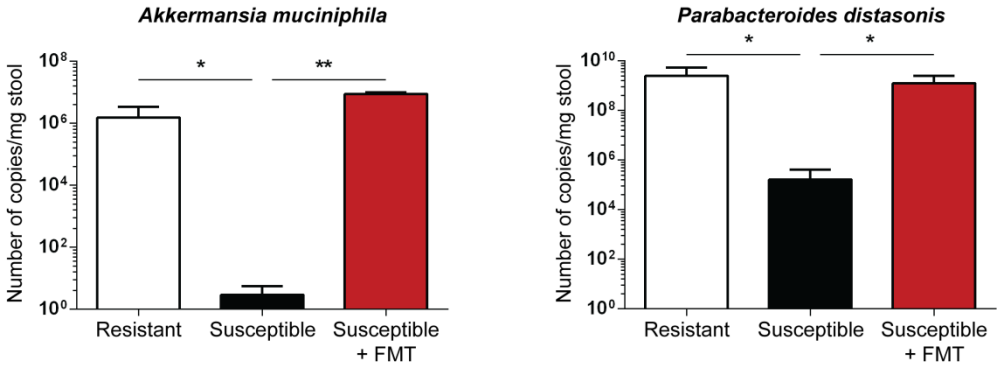
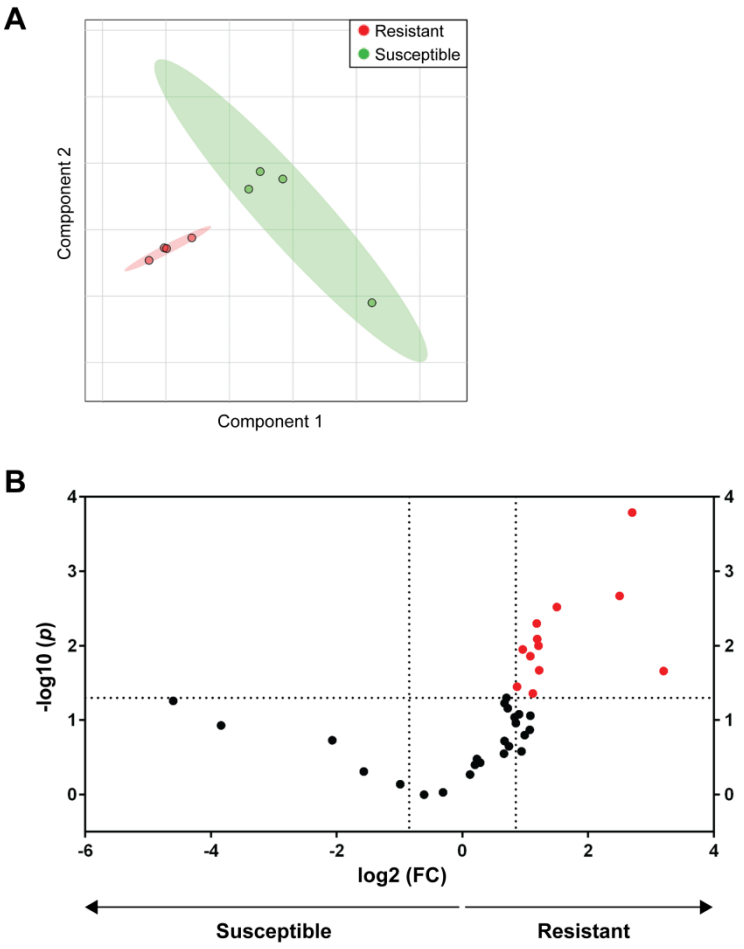


Figure II.9. *Akkermansia muciniphila* and *Parabacteroides distasonis* are significantly associated with a protective phenotype against colitis. (A) Most significant hits found in the metagenomic analysis, based on *p* value (<0.05) and adjusted *p* value (false discovery rate (FDR) <0.2); comparison is made by resistant *versus* susceptible mice and susceptible + FMT *versus* susceptible mice. **(B)** Absolute abundance quantification of *A. muciniphila* and *P. distasonis* in susceptible, resistant and susceptible + FMT mice. Images are representative of at least three independent experiments; n=5/6 per group. Data is presented as mean ± standard deviation (SD). Statistically significant values are: $*p < 0.05$; $**p < 0.01$.

Resistant phenotype is associated with a specific metabolic signature

The intestine offers a very particular metabolic environment that is known to impact immune cell development and function [3, 34]. To characterize the colon metabolic profile of both resistant and susceptible, colonic extracts of mice in homeostatic conditions were analyzed by NMR. Both groups clustered separately in a multivariate analysis of the metabolomic data (Figure II.10A). Indeed, from the 37 metabolites that were identified by the NMR analyses, 12 of them were significantly increased in resistant mice, with lactate being the most significant hit (Figure II.10B-C). Moreover, all the 12 metabolites found to be increased in the colon of resistant mice were not increased in the serum of the same mice, suggesting that these metabolites derive or act

specifically in the intestinal environment but are not drained systemically. Given that most metabolites present in the gut are derived from bacterial metabolism, this result imply that the two groups of mice display a distinct intestinal environment, which may in turn influence the susceptibility for colitis development. Therefore, it will be relevant to unveil if the metabolites identified in resistant mice are being produced or somehow derived from *A. muciniphila* and/or *P. distasonis*. The analysis of these metabolites may contribute to understand the mechanisms by which the identified microbiota candidates modulate intestinal protection. Moreover, by profiling the metabolic environment of susceptible mice, prior and after colitis development, allowed to identify which metabolites are altered during disease. This topic will be further addressed in Chapter III.



C

Metabolites	*p* value
Lactate	0.00016
Succinate	0.00213
Uracil	0.00299
Adenine	0.00498
Tyrosine	0.00820
Uridine	0.00995
Leucine	0.01112
Phenylalanine	0.01122
Nicotinic acid	0.01391
Formate	0.02138
Isoleucine	0.03545
AMP	0.04385

Figure II.10. Resistant mice displayed a specific metabolic profile. (A) Partial least squares – discriminant analysis (PLS-DA) was obtained for resistant and susceptible mice in homeostatic conditions. **(B)** Important metabolites selected by volcano plot with fold change threshold 1.8 and *p* value (t-test) threshold of 0.05. Red circles represent statistically significant metabolites. Both fold changes and *p* values are log transformed. **(C)** Metabolites identified as significantly different between the two groups of mice and respective *p* value. All metabolites were found

increased in resistant mice when compared to the susceptible group. AMP – adenosine monophosphate. Images are representative of one representative experiment out of three; n=10 per group.

A. muciniphila induces the expression of E-cadherin in epithelial cells in an *in vitro* gut-on-a-chip model

We have found that an enrichment in *A. muciniphila* and *P. distasonis* in the microbiota composition of mice is associated with protection against colitis development. Nevertheless, whether this protective phenotype is defined by the presence of these bacteria or only by one of them, and how this can dampen inflammation through the modulation of the epithelial barrier and immune response, needs to be further explored. By using an *in vitro* gut-on-a-chip model we are able to test the effect of these bacterial candidates in the epithelial barrier and therefore aim to unveil the mechanisms that can be triggered to induce protection against gut inflammation.

The gut-on-a-chip model used in this work consists in an *in vitro* system containing human colon epithelial cells (Caco-2 cell line) and intestinal microvascular endothelial cells (HUVECs) that form a basic organ structure simulating the intestinal layers. Additionally, macrophages are also embedded in the endothelial side of the system (Figure II.11A). The degree of precision of this platform is assured by the mechanostimulatory input obtained by the oscillating change of hydrostatic pressure that mimics the peristaltic movements of the gut and allows a proper three-dimensional cellular growth (Figure II.11B). This system also allows evaluating the expression of several proteins associated with epithelial barrier integrity and intestinal function, such as claudins and mucins (Figure II.11C), and the quantification of metabolites and inflammatory mediators, such as cytokines. Therefore, it represents a valuable tool to study the retrieved candidates above described.

Despite the gut-on-a-chip model is completely developed, this system was not optimized at the time for the use of anaerobic bacteria as components of the microbial environment of the gut. Although fully assembled chips were performed to get acquainted and test the full system in the future, for optimization purposes only epithelial cells were used and the effect of bacteria in the epithelial layer was assessed. This was mostly because we needed to use low oxygen concentrations and HUVECs are very sensitive to oxygen deprivation (preliminary observation). Hence, Caco-2 cells were seeded in the biochip and perfused for seven days, so that they could develop three-dimensional structures with similarity to human gut. Then, the oxygen concentration was gradually reduced to allow the cells to adjust to new hypoxic conditions. *A. muciniphila* and *P. distasonis* were then added to the biochip, alone or in combination (Figure

II.11D). The effect on epithelial cell layer was assessed by immunofluorescence staining of E-cadherin after 24 hours of incubation with bacteria. We observed that the presence of *A. muciniphila* leads to an increased expression of E-cadherin in epithelial cells incubated alone or in combination with *P. distasonis*. E-cadherin is a type of cell adhesion molecule that is involved in the formation of adherens junctions and subsequently for intestinal epithelial barrier function. Interestingly, this increased expression seems to be only dependent of *A. muciniphila*, since cells incubated with *P. distasonis* display similar expression of E-cadherin as control cells (without bacteria; Figure II.11E). Even though these data needs further confirmation, it is in accordance to what was found *in vivo* (Figure II.2C), demonstrating that *A. muciniphila* is a potential bacterial candidate for modulation of intestinal epithelial barrier and possibly be at the basis of the protective phenotype.

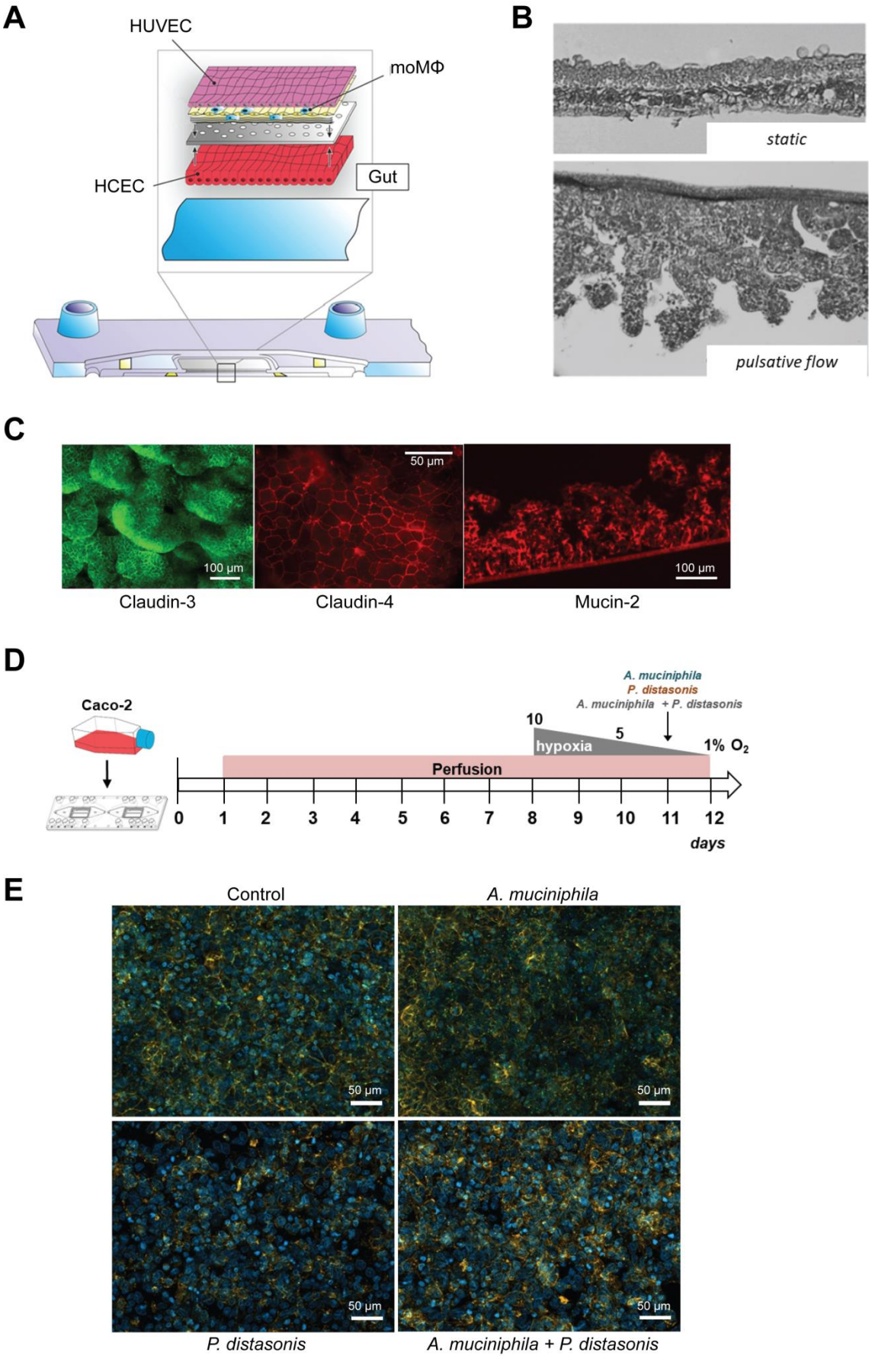

A
HUVEC
moMΦ
HCEC
Gut
B
static
pulsative flow
C
50 µm
100 µm
100 µm
Claudin-3
Claudin-4
Mucin-2
D
A. muciniphila
P. distasonis
A. muciniphila + P. distasonis
Caco-2
10
5
hypoxia
1% O2
Perfusion
0 1 2 3 4 5 6 7 8 9 10 11 12
days
E
Control
A. muciniphila
50 µm
50 µm
50 µm
50 µm
P. distasonis
A. muciniphila + P. distasonis

Figure II.11. The gut-on-a-chip model as a powerful tool to study the role of bacterial microbiota candidates in intestinal homeostasis and disease. (A) Graphic representation of a biochip used in this work; HUVEC – human umbilical vein endothelial cells; moMØ – monocyte-derived macrophages; HCEC – human colonic epithelial cells. **(B)** Microscopical evaluation of epithelial cell development in a static or perfused system. **(C)** Expression of claudin-3, claudin-4 and mucin-2 in epithelial cells from a perfused biochip assessed by immunofluorescence (data provided by Alexander Mosig's research group). **(D)** Schematic of the *in vitro* gut-on-a-chip evaluation of the impact of *A. muciniphila* and *P. distasonis* in epithelial cells. **(E)** E-cadherin expression (yellow) in epithelial cells in the several tested conditions; counterstained with DAPI. Images are representative of one experiment.

DISCUSSION

The maintenance of intestinal homeostasis is crucial to prevent exacerbated immune responses to commensal bacteria, and therefore a tight regulation of the cellular mechanisms by which homeostasis is achieved is needed [35, 36]. The balance between the gut microbiota, the immune system and the intestinal epithelial barrier must then be preserved; nonetheless, the mechanisms by which these three components of the intestinal environment can impact one another are not fully understood. Yet, disruption in this dynamic is definitely responsible for the development of intestinal inflammatory disorders [13]. Therefore, one of the main challenges in the understanding the immunopathology of IBD (in particular, of CD and UC) is the fact that we are facing a multifactorial disorder in which it is not clear what can be cause or consequence of the disease.

In this work we have found that mice from different animal facilities have different susceptibility to DSS-induced colitis, with one of the groups displaying a notable protection against disease development. This unforeseen protective phenotype seems to be directly linked with the microbiota composition of the two groups of mice. The characterization of the intestinal microbiota of susceptible and resistant mice revealed that both groups displayed a distinct microbiota profile, with *A. muciniphila* and *P. distasonis* found significantly increased in resistant mice. *A. muciniphila* is a Gram-negative, anaerobic, mucus-colonizing bacterium that degrades mucin, leading to the production of propionate and acetate, and several studies have pointed its impact in the modulation of host intestinal epithelial genes involved in basal metabolism [37, 38]. Moreover, the abundance of *A. muciniphila* was decreased in patients with IBD and other pathologies such as obesity and type 2 diabetes [39-42]. *P. distasonis* is a Gram-negative, anaerobic bacterium belonging to *Parabacteroides* genus, which presents conflicting reports regarding its role in IBD, being both associated with enhanced or attenuated colitis in mice [43, 44]. Similar contradictory results for *P. distasonis* were also found in IBD patients [45, 46]. In this work, these bacteria seem to be strongly associated with protection against colitis, since susceptibility to colitis was abrogated by the transfer of fecal contents from enriched in *A. muciniphila* and *P.distasonis*, in both acute and chronic models of colitis.

The protective phenotype was also found to be associated with an overexpression of mucin-encoding genes and other genes encoding for proteins associated with gut epithelial barrier. These proteins are involved in the maintenance of a proper epithelial barrier function and

alterations in these proteins are reported in IBD patients [23]. Nevertheless, the dissimilar expression of these proteins in susceptible and resistant mice were found in homeostatic conditions, which may suggest at least that these two groups of mice shown a different predisposition or activation of the epithelial barrier to respond to an inflammatory challenge. The expression of *MUC2* is widely described as essential for goblet cell function and mucus layer formation, and impairments in this gene are associated with colitis development [24]. Interestingly, not only resistant mice have shown increased expression of mucin-encoding genes, but also in higher number of goblet cells in homeostatic conditions. Indeed, even upon colitis induction resistant mice maintained similar amounts of goblet cells, contrary to the susceptible ones, in which severe crypt ablation was observed, with fewer goblet cells remaining intact. Actually, the higher number of goblet cells present in resistant mice is in accordance with the colonization by *A. muciniphila*, since it is described that this bacterium stimulates host mucin production and increase of the mucus layer thickness, with a possible role in reinforcing the intestinal barrier function [37, 38, 47]. This supports the premise that the epithelial barrier of these mice is more prone to sustain an inflammatory insult.

In accordance with this hypothesis are the increased levels of IL-10, IL-17 and IL-22 found in resistant mice under homeostatic conditions. While the role of IL-10 in the gut is well established [48, 49], the expression of IL-17 and IL-22 is interesting due to their plastic behavior [30]. IL-17 is a pro-inflammatory cytokine that is extensively associated with disease, although anti-IL-17 has failed as immunotherapy for colitis, showing that this cytokine plays also an important immunoregulatory function [50, 51]. Indeed, a recent study has shed some light in this dual role of IL-17, showing that the blockade of IL-17F, but not of IL-17A, conferred protection against colitis by inducing Treg cells via intestinal microbiota [52]. Since we have quantified total IL-17, we will further dissect if the increased amount of this cytokine in resistant mice is mostly dependent on A or F isoforms. Our results also seem to indicate that other cell types besides Th17 cells and ILC3 may be responsible for the increased levels of IL-17 found in the intestine of resistant mice. For instance, gamma delta ($\gamma\delta$) T cells are IL-17-producing cells that are highly represented in epithelial barrier tissues, playing an important role in barrier surveillance and in tissue repair and homeostasis [53]. Therefore, further characterization of the cell populations that can be contributing for this specific intestinal environment must be performed. Finally, IL-22 is involved in epithelial cell protection by the expression of antimicrobial peptides, tissue

regeneration and also promotes intestinal wound healing from acute intestinal injury, contributing therefore for the maintenance of intestinal epithelial barrier [54-56].

We have shown that an enrichment in *A. muciniphila* and *P. distasonis* seems to have a protective role in mice by sustaining inflammation and inhibiting colitis development, most likely by impacting intestinal epithelial barrier to better tolerate an inflammatory event and injury. Although there are several reports already focused on the impact of these bacteria in the intestinal homeostasis, some of them shown contradictory results. Hence, it is paramount to understand how these bacteria can influence intestinal function. The supplementation of susceptible mice with *A. muciniphila* and *P. distasonis*, alone or in combination, followed by colitis induction, will be performed to corroborate the previous results. The specific mechanisms that can be underlying this protection are currently being explored in a biochip-based human gut model that contemplates the complexity of signaling and function within the intestinal epithelium. With this approach it will be possible to test not only the bacterial candidates, alone or in combination, that are associated with an increased epithelial barrier integrity, but also to evaluate the inflammatory mediators and metabolites derived by the presence of these bacteria in the intestinal context. The expression of proteins associated with epithelial barrier function can also be evaluated using the gut-on-a-chip platform. Indeed, preliminary results have shown that the incubation of *A. muciniphila* in biochips containing epithelial cells leads to an overexpression of E-cadherin in these cells, which is in accordance to what we observed in the colon of resistant mice. Thus, by using this innovative *in vitro* model, we are able to adjust culture conditions and obtain a detailed investigation of the molecular and cellular processes involved in the mediated immune response and regulation of the intestinal epithelial barrier. The most relevant findings will then be explored in several intestinal inflammatory contexts, such as chemical and spontaneous murine models of IBD.

Hence, in this work we described a protective phenotype against colitis found in a group of mice, which we associate with a more tolerant intestinal epithelial barrier to sustain an intestinal insult. The future work within this project will clarify the microbiota partners and its derived factors that promote intestinal epithelial barrier integrity with increased tolerance to external damage. We aim that the findings will provide new insights regarding the interaction between the microbiota, epithelial barrier and immune response in the gut and may hold promise for the development of innovative therapies and clinical approaches for IBD.

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CHAPTER III

L-THREONINE SUPPLEMENTATION DURING COLITIS ONSET DELAYS DISEASE RECOVERY

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L-Threonine supplementation during colitis onset delays disease recovery

Joana Gaifem, Luís G. Gonçalves, Ricardo J. Dinis-Oliveira, Cristina Cunha, Agostinho Carvalho, Egídio Torrado, Fernando Rodrigues, Margarida Saraiva, António G. Castro and Ricardo Silvestre

IN BRIEF

Supplementation of mice with L-Threonine in the beginning of colitis development has shown to be detrimental for the remission of the disease and it is associated with a remarkable decrease in goblet cell number.

HIGHLIGHTS

- Minor changes were observed upon a metabolomic analysis of colonic extracts performed during colitis development.

- Among those, L-Threonine concentration on colonic extracts was found to be significantly altered with a twofold reduction during acute colitis.

- Threonine supplementation during colitis onset decrease disease recovery associated with a reduced number of goblet cells in the gut crypts and decreased expression of *Muc2* and IL-22 secretion.

- Threonine supplementation upon the establishment of inflammation did not impact disease development and recovery, or any other measured parameter of epithelial barrier stability.

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BRIEF RESEARCH REPORT
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L-Threonine Supplementation During Colitis Onset Delays Disease Recovery

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Dietary nutrients have emerged as potential therapeutic adjuncts for inflammatory bowel disease (IBD) given their impact on intestinal homeostasis through the modulation of immune response, gut microbiota composition and epithelial barrier stability. Several nutrients have already been associated with a protective phenotype. Yet, there is a lack of knowledge toward the most promising ones as well as the most adequate phase of action. To unveil the most prominent therapy candidates we characterized the colon metabolic profile during colitis development. We have observed a twofold decrease in threonine levels in mice subjected to DSS-induced colitis. We then assessed the effect of threonine supplementation in the beginning of the inflammatory process (DSS + Thr) or when inflammation is already established (DSS + Thr D8). Colitis progression was similar between the treated groups and control colitic mice, yet threonine had a surprisingly detrimental effect when administered in the beginning of the disease, with mice displaying a delayed recovery when compared to control mice and mice supplemented with threonine after day 8. Although no major changes were found in their metabolic profile, DSS + Thr mice displayed altered expression in mucin-encoding genes, as well as in goblet cell counts, unveiling an impaired ability to produce mucus. Moreover, IL-22 secretion was decreased in DSS + Thr mice when compared to DSS + Thr D8 mice. Overall, these results suggest that supplementation with threonine during colitis induction impact goblet cell number and delays the recovery period. This reinforces the importance of a deeper understanding regarding threonine supplementation in IBD.

Keywords: IBD, threonine, DSS-induced colitis, goblet cells, metabolomics, IL-22, mucin

INTRODUCTION

Inflammatory bowel disease (IBD) is a complex debilitating disorder of the gastrointestinal tract which comprises both Crohn's disease and ulcerative colitis. Despite the unclear etiology of IBD, several factors have been accounted as key for the development of the disease, such as genetics, immune system and environmental factors, namely diet and gut microbiota composition (Khor et al., 2011).

Dietary supplementation has emerged as a promising therapeutic practice in the prevention and treatment of IBD (Durchschein et al., 2016). Recent evidence has revealed that fiber-enriched diets promote protection against IBD development, since dietary fiber is mainly fermented by intestinal microbiota into short-chain fatty acids (SCFAs), such as butyrate, acetate and propionate (den Besten et al., 2013). The protective properties of these metabolites are widely described by their impact on immune cell activation and epithelial barrier stability (Kelly et al., 2015; Macia et al., 2015), with decreased levels of SCFAs being found in colon samples from IBD patients (Huda-Faujan et al., 2010). Other studies have also pointed out several specific amino acids that can improve intestinal homeostasis, mainly by boosting mucosal healing and regeneration. For instance, glutamine is known to promote protection in dextran sulfate sodium (DSS)- and 2,4,6-trinitrobenzenesulfonic acid (TBNS)-induced intestinal inflammation, acting via NF-κB downregulation (Kretzmann et al., 2008). Colitic mice orally administered with glutamine displayed suppressive Th1/Th17 immune responses and subsequently decreased inflammation when compared to mice fed with regular diet (Hsiung et al., 2014). Other amino acids have been also associated with a protective phenotype against colitis. Using distinct animal models of colitis, diets enriched in threonine, serine, proline and cysteine, given before and throughout disease development, have been shown to restore mucin synthesis and stabilization of gut microbiota (Faure et al., 2006). Similar findings were observed with the administration of a mixture of threonine, methionine and monosodium glutamate after colitis induction (Liu et al., 2013).

Several studies have so far addressed whether administration of specific nutrients may arise as a prophylactic and/or therapeutic approach. However, there is a lack of knowledge toward the most promising and adequate phase of action. Thus, we investigated the metabolic profile of mice developing colitis, aiming to identify variation of metabolites during inflammation. The identification of the most attractive potential targets for therapy and the definition of a time range more prone to potentiate the effects of their supplementation may be relevant for future applications in IBD prophylaxis and therapy.

MATERIALS AND METHODS

Animals

Seven to nine-week old C57BL/6J male mice were purchased from Charles River Laboratories and housed in i3S animal facilities, under pathogen free conditions, with food and water *ad libitum*. All experimental procedures were approved by the i3S Animal Ethics Committee and licensed by the Portuguese National Authority for Animal Health (DGAV) with reference 014811/2016-07-13.

Colitis Induction

Dextran sulfate sodium (DSS; TdB Consultancy; 2% (w/v), molecular weight approximately 40000 Da) was administered in drinking water *ad libitum* for 5 days. Clinical signs of colitis were monitored daily and scored as a disease activity index (DAI; **Supplementary Table S1**).

L-Threonine Administration

Mice were divided into DSS (control), DSS with L-Threonine (DSS + Thr) and DSS followed by L-Threonine administration at day 8 (DSS + Thr D8), as shown in **Figure 2A**. L-Threonine [Thr; Sigma–Aldrich; 0.166% (w/v) corresponding to 250 mg/Kg/day] was given in the drinking water *ad libitum*. The dose was chosen according to the daily intake in previous studies with rodents (Faure et al., 2006; Liu et al., 2013) and to a high-threonine human supplementation study (Pencharz et al., 2008). The safety of our protocol measuring biomarkers of renal and liver damage was evaluated to confirm the absence of toxicity (**Supplementary Figure S1**). Similar fluid intake was found among all groups.

Metabolomic Analysis by Nuclear Magnetic Resonance (NMR)

Methanol/water extracts of colon were analyzed at an UltrashiedTM 800 Plus (Bruker) spectrometer as described in Graça et al. (2017). Metabolite concentrations were performed by integration of 1H-NMR resonances using TSP as reference.

Quantitative Real-Time PCR (qPCR)

Total RNA was isolated from colonic samples (TripleXtractor, Grisp). As DSS inhibits both polymerase and reverse transcriptase activities, RNA was purified with lithium chloride, as in Viennois et al. (2013). qPCR was performed as described in Correia et al. (2017). The list of primers used is in **Supplementary Table S2**.

Histology and Goblet Cell Count

Colons were fixed in 10% buffered formalin (Sigma–Aldrich) and embedded in paraffin. Sections of 5 μm were stained with hematoxylin/eosin and Alcian Blue/Periodic acid-Schiff. Goblet cell number was assessed for each experimental condition in a blinded fashion. Only crypts cut longitudinally from crypt opening to bottom were analyzed.

Cytokine Quantification

Colonic explant cultures were performed as previously described (McNamee et al., 2011). Cytokine quantification was performed in supernatants by ELISA (Biolegend). Tissue explants were homogenized and total protein was measured using Bradford assay. The concentration of secreted cytokines in the supernatant was normalized to total tissue protein and expressed as picogram of cytokine *per* μg of total tissue protein.

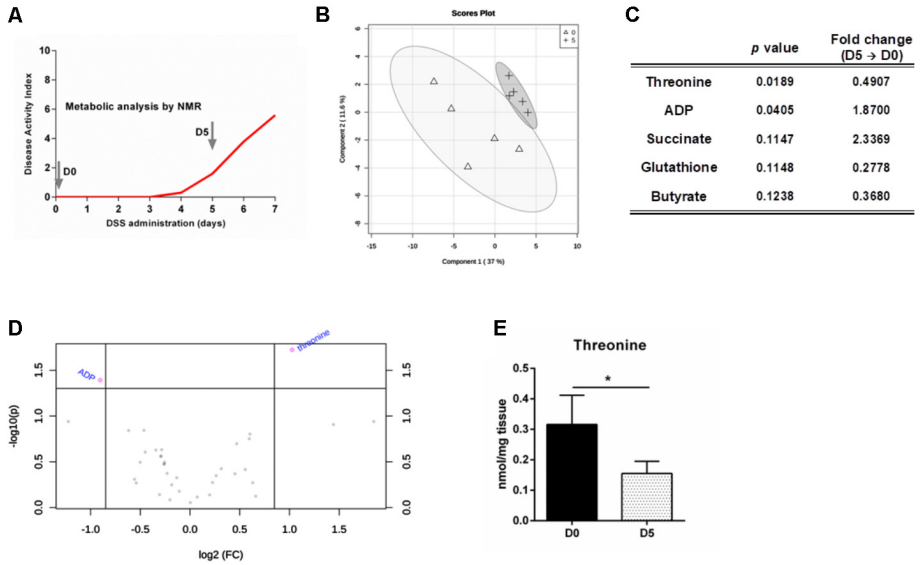

FIGURE 1 | Threonine levels in the colon decrease with intestinal inflammation. **(A)** Mice were treated with DSS to induce colitis and colon metabolites were measured at day 0 and day 5 by NMR. **(B)** Partial least squares – discriminant analysis (PLS-DA) was obtained for samples of both days. **(C)** Top-five features identified by the combination of the *p*-value from *t*-test analysis and magnitude of the change (fold change from day 5 *versus* day 0). **(D)** Important metabolites selected by volcano plot with fold change threshold (x) 1.8 and *t*-test threshold (y) of 0.05. The purple circles represent features above the threshold. Both fold changes and *p*-values are log transformed. **(E)** Threonine levels in the colon at day 0 and day 5. Data is shown as mean ± SD; $n = 5$ mice/group. One representative experiment is shown out of two. $^{*}p < 0.05$.

Statistical Analysis

Metabolite differences were evaluated by ANOVA in R statistical software. Partial least squares – discriminant analysis (PLS-DA) models were performed in SIMCA software. Other statistical analyses were carried out with GraphPad Prism (version 6.01). For multiple group comparisons one-way ANOVA test with a Tukey multiple-comparison posttest was performed, while for multiple group comparisons with repeated measures two-way ANOVA test with a Tukey multiple-comparison posttest was applied. Data are presented as mean ± standard deviation (SD). Statistically significant values are: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

RESULTS AND DISCUSSION

To characterize the colon metabolic profile during colitis development, metabolites present on colonic extracts of mice prior (day 0) and after 5 days of DSS-induced colitis were analyzed by NMR (**Figure 1A**). These time points were selected since it allows the comparison of a homeostatic profile (day 0) against a period with established inflammation and lesion, yet reversible and treatable (day 5). Multivariate analyses of the metabolomic data only demonstrated minor alterations between profiles for day 0 and day 5 of colitis development (**Figure 1B**). However, univariate analyses performed on the metabolites allow to discriminate ADP and particularly threonine as significantly altered from day 0 to day 5 (**Figures 1C,D**). Among essential amino acids, threonine has a prominent role in maintaining a healthy gut. Threonine is able to generate the main three SCFAs, namely acetate, butyrate and propionate (Neis et al., 2015). In fact, it has been previously identified several biosynthetic genes for threonine metabolism in the human gut microbiota, suggesting the relevance of this amino acid for microbiota biology (Abubucker et al., 2012). SCFAs are described as important modulators of immune response, since they are ligands for G-protein-coupled receptor 43 (GPR43) that is expressed by immune cells on the lamina propria, such as regulatory T cells, regulating the proinflammatory responses in the intestine (Bollrath and Powrie, 2013). Moreover, threonine is vastly metabolized in the intestine for mucin synthesis (Faure et al., 2005). These proteins are paramount in intestinal stability, since the mucus layer in the colonic outer layer prevents the direct contact of luminal microorganisms with the epithelium (Johansson et al., 2014). It has also been suggested that threonine requirements are increased under pathological settings to maintain proper intestinal function, such as production and formation of the mucus layer (Remond et al., 2009). Therefore, by participating in the mucus layer synthesis and production of anti-inflammatory SCFAs, threonine metabolism by gut microbiota proves to be essential for gut barrier integrity and function. Our data show that threonine levels drop 2-fold during colitis development until day 5 (**Figures 1C–E**). Therefore, we hypothesized that threonine supplementation during active inflammatory disease could help to restore the intestinal homeostasis and thus present some therapeutic potential.

Previous studies have investigated threonine in combination with other amino acids as a potential candidate for therapy against colitis (Faure et al., 2006; Liu et al., 2013). Nevertheless,

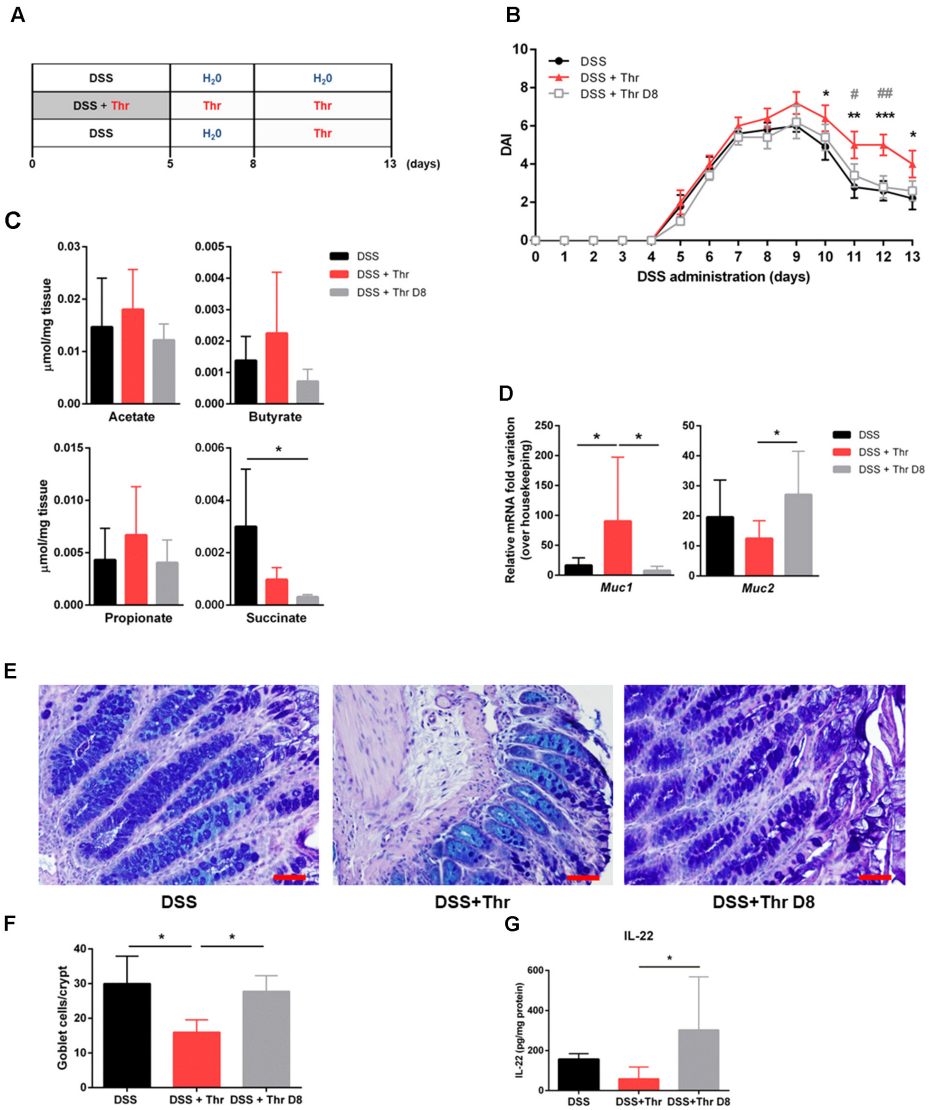

FIGURE 2 | Threonine supplementation on the onset of colitis development delays recovery and decreases goblet cell number. **(A)** Mice were supplemented with threonine in the beginning of colitis induction (DSS + Thr) or after established inflammation (DSS + Thr D8). Mice treated only for colitis induction were used as control (DSS). **(B)** Disease progression was assessed by scoring the disease activity index (DAI) throughout the experiment. **(C)** Quantification of short-chain fatty acids (acetate, butyrate, propionate) and succinate by NMR was performed at day 13 of experiment. **(D)** Expression of mucin-encoding genes *Muc1* and *Muc2* was performed by qPCR at day 13. **(E)** Colonic tissue sections were stained with Alcian Blue/periodic acid-Schiff. **(F)** Quantification of goblet cell number per crypt. Scale bar = 50 µm. **(G)** IL-22 quantification on colonic explant cultures by ELISA. Data is shown as mean ± SD. $n = 5$ mice/group. One representative experiment is shown out of two. The symbol * corresponds to statistical differences between DSS + Thr and DSS; while # is related to statistical differences between DSS + Thr and DSS + Thr D8. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

not only single threonine supplementation was not evaluated before, but also there is scarce evidence regarding the most adequate time frame for its supplementation in colitis treatment. Accordingly, we addressed threonine supplementation in two distinct phases: (1) in parallel with the initiation of the inflammatory process, i.e., simultaneously with DSS administration (DSS + Thr), and (2) on day 8, when inflammation is already established (DSS + Thr D8). Mice subjected to DSS-induced colitis but without threonine supply were used as control (DSS) (**Figure 2A**). We observed that the three groups developed colitis with a similar progression profile. Nevertheless, after this time point, the recovery profile of colitic mice supplemented with threonine in the drinking water (DSS + Thr) was slower than that of the other two groups, showing statistically significant differences from day 10 to the final day of experiment when compared to control group (DSS). Besides, DSS + Thr mice had also a distinctive DAI score at day 11 and 12 when compared with mice that only received threonine after day 8 (DSS + Thr D8) (**Figure 2B**). Despite the divergent

phenotype, no major differences were found in colon length neither in the intestinal permeability (**Supplementary Figure S1**).

Threonine administration was surprisingly detrimental for disease recovery when given at the setting of the inflammatory process. To understand this phenotype, we first evaluated the potential alterations in the colon metabolic profile among the different groups. No significant differences were found between most of the metabolites. Particularly, the levels of the major SCFAs (i.e., acetate, butyrate, and propionate), normally associated with a protective phenotype, were similar among groups. Only succinate levels, which is as an important marker of inflammation promoting IL-1β induction in inflammatory contexts (Tannahill et al., 2013), were found to be markedly decreased in DSS + Thr D8 mice (**Figure 2C**). No major alterations were observed in the inflammatory infiltrate profile, tissue organization and hepatic and renal toxicity serum biomarkers (**Supplementary Figure S1**).

Threonine plays a major role in mucin synthesis and consequently in the formation of the mucus layer. Indeed, lack of threonine is also known to impair intestinal paracellular permeability and is associated with fewer goblet cells and mucus synthesis (Faure et al., 2005; Mao et al., 2011). The mucus layer serves as a barrier against microbial translocation to the lamina propria and therefore its integrity is paramount for intestinal homeostasis. When we analyzed the expression of mucin-encoding genes, we found that *Muc2* expression is decreased in DSS + Thr mice when compared to mice that only received threonine at day 8 (DSS + Thr D8) (**Figure 2D**). *Muc2* encodes for the oligomeric mucus gel-forming mucin 2 protein that is the major responsible for mucus synthesis. Indeed, impairment or total absence of the mucus layer is associated with severe colitis, as observed in *Muc2*-deficient mice (Van der Sluis et al., 2006). We also found that DSS + Thr mice display higher expression of *Muc1*, which has shown to contribute to intestinal inflammation and colon cancer progression (Baldus et al., 2004; Takahashi et al., 2015). We next quantified the number of goblet cells of the colonic mucosa. These are a secretory epithelial cell lineage found in both the small and the large intestines, whose major function is the production of mucus. By analyzing colon slices stained with Alcian Blue/Periodic acid-Schiff, we observed that DSS + Thr mice displayed significantly fewer goblet cells when compared to both DSS and DSS + Thr D8 (**Figures 2E,F**). Therefore, our results suggest that an alteration in mucus synthesis due to threonine administration during the onset of disease may impact intestinal integrity, by delaying the recovery of disease. Faure et al. (2006) have demonstrated that supplementation with diet containing higher doses of amino acids, including L-threonine, lead to an increase in goblet cell number, regulated mucin production in the colon and restored microbiota composition after DSS treatment in rats. Notwithstanding, not only the animal model is different, but also L-threonine was given before colitis induction, which may be underlying the distinctive outcome.

Previous studies have linked several cytokines to mucus production in the intestine (Parks et al., 2015). To examine the immunological profile of the three groups, cytokine levels in the colon were quantified. No major changes were observed between the groups for interleukin (IL)-1β, IL-12p70, IL-10, IL-17A/F and granulocyte-macrophage colony-stimulating factor (GM-CSF) levels (**Supplementary Figure S2**). Notwithstanding, the amount of IL-22 was significantly decreased in DSS + Thr mice when compared to DSS + Thr D8 mice (**Figure 2G**). IL-22 is a member of the IL-10 family of cytokines and has been vastly studied in the context of intestinal homeostasis. It can be produced by several cell types, such as T helper (Th1) 1, Th17, Th22 and innate lymphoid cells (ILCs), and present several roles in the gastrointestinal tract, such as tissue regeneration and maintenance of the intestinal epithelial barrier (Rutz et al., 2013). Thus, the decreased IL-22 levels may be associated with delayed recovery of the intestinal balance.

Overall, our data demonstrate that supplementation of threonine during colitis induction impairs goblet cell number, with concomitant decreased *Muc2* expression and IL-22 production. These variations are likely to be the cause of delayed recovery observed in this situation. Interestingly, these effects are not seen when threonine is administered once colitis is established. Acute DSS-induced colitis is known to promote gut microbial dysbiosis (Munyaka et al., 2016). Threonine is metabolized by some intestinal commensal bacteria, leading to the production of several metabolites used for intestinal maintenance and to mediate immune responses (Neis et al., 2015). Thus, threonine supplementation during induction of colitis may impact differently the colonic microbiota populations present during the onset and upon the establishment of inflammation, having ultimately distinct effects in intestinal function. Further understanding of the mechanisms underlying threonine supplementation may give new insights on how dietary nutrients modulate the dynamic balance between microbiome, immune response and barrier function.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of European Council Directive (2010/63/EU) guidelines that where transposed into Portuguese law (Decree-Law n.°113/2013, August 7th), i3S Animal Ethics Committee and licensed by the Portuguese National Authority for Animal Health (DGAV). The protocol was approved by the i3S Animal Ethics Committee and licensed by the Portuguese National Authority for Animal Health (DGAV) with reference 014811/2016-07-13.

AUTHOR CONTRIBUTIONS

JG, ET, CC, AgC, FR, MS, AnC, and RS designed the experiments. JG, LG, RD-O, and RS performed the experiments. JG, LG, RD-O, and RS analyzed the data. JG, LG, RD-O, and RS interpreted the results. JG and RS drafted the manuscript and prepared the tables and figures. JG, LG, RD-O, ET, CC, AgC, FR, MS, AnC, and RS revised the paper and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2018.01247/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer DS and handling Editor declared their shared affiliation at the time of the review.

Supplementary methods

In vivo intestinal permeability assay

To assess barrier function at day 13, *in vivo* intestinal permeability assay was performed by FITC-labelled dextran administration. Food and water were withdrawn for eight hours and mice were administered with 44 mg/100 g body weight of FITC-labelled dextran (TdB Consultancy; 4 kDa) by oral gavage. Serum was collected four hours later and fluorescence intensity was measured by spectrophotofluorimetry (excitation: 485 nm; emission: 528 nm). Concentrations were determined using a standard curve of serially diluted FITC-labelled dextran. Serum from mice not administered with the permeability tracer was used as background control.

Quantification of serum toxicological biomarkers

Aspartate transaminase (AST), alanine aminotransferase (ALT) and creatinine were measured on an AutoAnalyzer (PRESTIGE 24i, PZ Cormay S.A.).

Supplementary Tables and Figures

S1 Table – Disease activity index (DAI) scores for colitis evaluation. The final score is obtained by the sum of each parameter.

Score	Weight loss	Stool consistency	Bleeding
0	No loss	Normal	No blood
1	1-5%	Mild-soft	Brown color
2	6-10%	Very soft	Reddish color
3	11-20%	Diarrhea	Bloody stool
4	> 20%		Gross bleeding

S2 Table – List of primers used for qPCR.

Genes	Forward sequence	Reverse sequence
Muc1	CCCTATGAGGAGGTTTCGGC	AAGGGCATGAACAGCCTACC
Muc2	TCCTGACCAAGAGCGAACAC	ACAGCACGACAGTCTTCAGG
Ubq	TGGCTATTAATTATTCGGTCTGCAT	GCAAGTGGCTAGAGTGCAGAGTAA

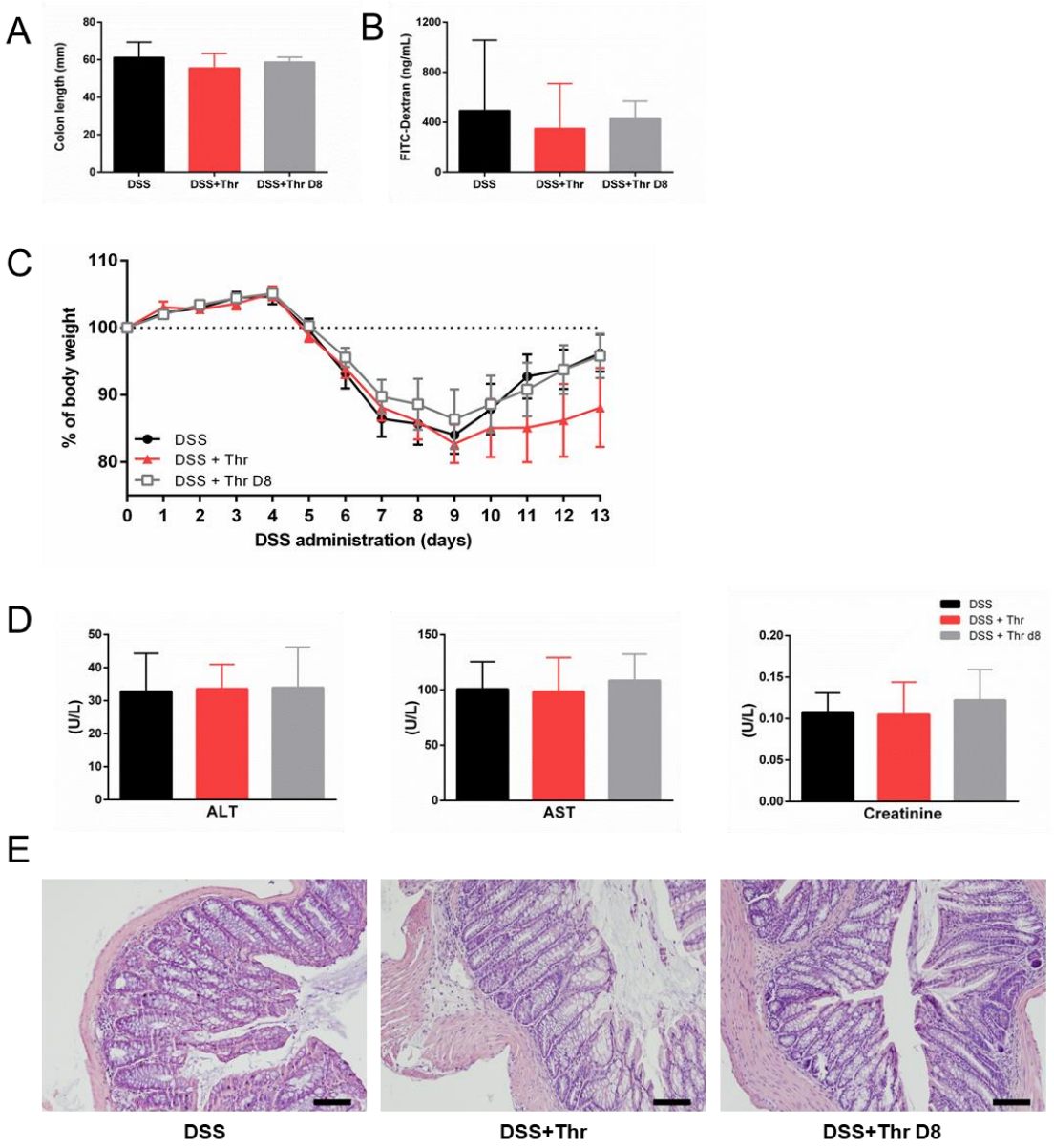

Figure S1 - Evaluation of histological and clinical parameters. (A) Colon length was measured after excision at day 13. (B) Intestinal permeability was measured after administration of FITC-Dextran by oral gavage and quantified in the serum after four hours of administration. (C) As one of the parameters of DAI, the body weight was measured daily during the experimental procedure. (D) Serum levels of alanine transaminase (ALT), aspartate aminotransferase (AST) and creatinine were analyzed for renal and hepatic toxicity control. (E) Histological analysis of hematoxylin & eosin staining of mice treated with DSS, DSS+Thr and DSS+Thr D8. Scale bar = 100 µm. Data is shown as mean ± SD; n=5 mice/group. No statistical differences were found between conditions.

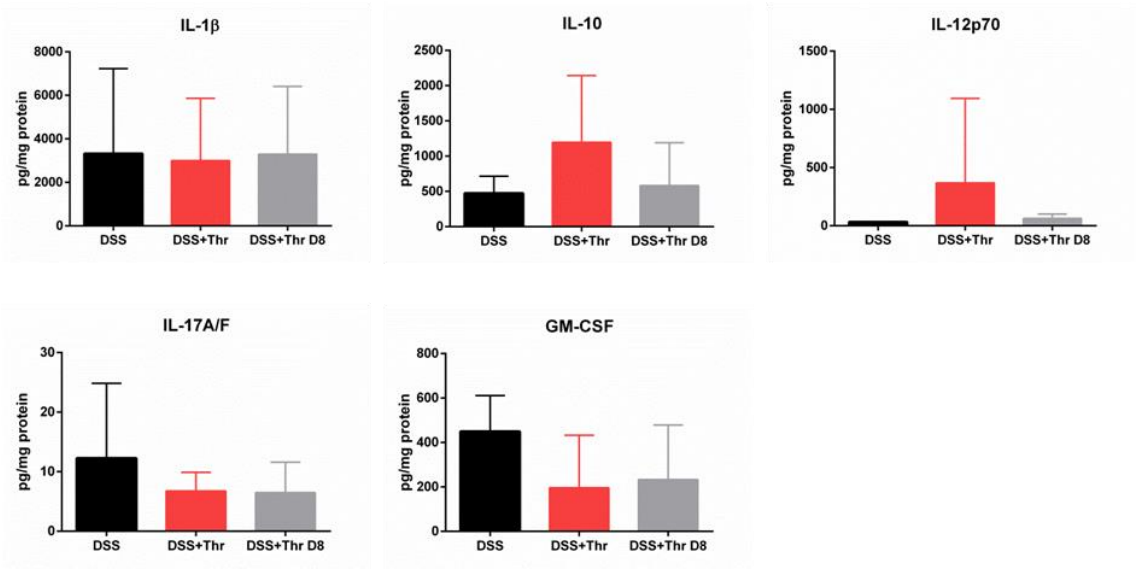

Figure S2 - Quantification of cytokine levels in supernatants from colonic extracts. Levels of colonic IL-1β, IL-10, IL-12p70, IL-17A/F and GM-CSF were measured in the supernatants of ex vivo colonic explants cultured at day 13. The concentration of secreted cytokines in the supernatant was normalized to total explant protein and expressed as picogram of cytokine per milligram of protein. Data is shown as mean ± SD; n=5 mice/group. No statistical differences were found between conditions.

CHAPTER IV

GENERAL DISCUSSION

The data presented in this thesis address two complementary topics that can provide groundbreaking advances in the comprehension of the mechanisms underlying IBD. The first focuses on the impact of intestinal microbiota on the susceptibility to colitis and the mechanisms responsible for the regulation of intestinal homeostasis. We identified two bacterial candidates, *Akkermansia muciniphila* and *Parabacteroides distasonis*, which were found significantly increased in mice evidencing a remarkable protection against colitis induction. This protection is dependent on microbiota composition since the treatment of susceptible mice with fecal contents from resistant ones rescues the phenotype, with mice becoming protected from both acute and relapse-remission colitis models. Current findings point towards a microbiota-induced impact in the intestinal epithelial barrier that allows it to better sustain an inflammatory insult and therefore conferring protection against colitis.

The second topic is based on the effect of L-threonine supplementation in an IBD context. We explored the metabolic variations occurring in the gut during colitis development, and found a twofold decrease in L-threonine levels from the beginning of colitis induction until the day when the first symptoms of the disease occurred. The impact of L-threonine supplementation in colitis was then evaluated with two distinct approaches: mice were supplemented with L-threonine in the beginning of disease development or after the inflammation being established. We found that the supplementation with L-threonine in the beginning of colitis development is detrimental to the remission of the disease. On contrary, L-threonine supplementation upon established inflammation did not influence colitis recovery. Decrease in *Muc2* expression and IL-22 production combined with a reduced number of goblet cells in the intestine were found in mice treated with L-threonine during the onset of colitis.

The relevance of the topics presented above – the contribution of microbiota to health and disease, and the dietary supplementation to prevent, ameliorate or even treat IBD – is undeniable in the context of research in IBD. The impact of intestinal microbiota in diverging from protection to susceptibility and the potential of dietary supplementation in disease management and prevention is recognized among the scientific and medical communities; still, there is a lack of knowledge on how these factors can be used in the development of effective predictive biomarkers of susceptibility and/or disease and in the implementation of innovative therapeutic approaches. One of the main challenges in this field is therefore to understand how these elements are intertwined and how they can be modulated in favor of a healthy intestinal environment.

1. MICROBIOTA-MEDIATED REGULATION OF INTESTINAL HOMEOSTASIS

The intestinal microbiota has a decisive role in the maintenance of intestinal homeostasis by its contribution to immune cell training, the occupation of niches avoiding pathogen colonization, and the synthesis of vitamins, nutrients and other compounds only accessible through microbial metabolism [1]. In this sense, intestinal microbial populations must live in equilibrium so that the intestinal environment is not perturbed.

Despite several advances in IBD research, the etiology of this pathology is still unclear. Several non-mutually exclusive events can occur and contribute to the development of IBD: infection with a persistent pathogen; impairments in immune system by insufficient effector cell activation or lack of regulatory cell activity towards intestinal bacteria; abnormal permeability of the intestinal epithelial barrier allowing excessive microbial translocation; and imbalance between harmless and harmful commensal intestinal bacteria, i.e., dysbiosis [2].

Alterations in the biodiversity of the fecal microbiota are correlated with IBD development, such as the decrease in the abundance of bacteria within the dominant phylum Firmicutes [3]. For instance, Sokol and colleagues have found that a decrease in *Faecalibacterium prausnitzii*, one of the major members of Firmicutes, is associated with an increased risk of postoperative recurrence of ileal CD [4]. The supplementation of colitic mice with *F. prausnitzii* or its supernatant markedly decreased the severity of the disease and promoted the correction of the dysbiosis associated with TNBS-induced colitis. This protective phenotype seemed to derive from the higher amounts of butyrate produced by the bacterium, which block NF-kB activation and IL-8 production [4]. The reestablishment of the microbiota composition was recently shown to be pivotal for colitis management, as it was demonstrated by Wang and colleagues with a pioneer treatment of immune checkpoint inhibitors-associated colitis by FMT from healthy individuals [5]. This provides new challenging insights on how modulation of the gut microbiome can be effectively associated with the recovery of colitis. Nevertheless, it is important to uncover the specific mechanisms underlying the protective effects, since diverse factors can be at the genesis of the disease.

Reduction in the number and diversity of species in the gut is often associated with pathology [6]. Notwithstanding, we have described in Chapter II a group of mice that display a resistant phenotype against colitis, despite revealing a decrease in number and diversity of bacterial species in the gut. Indeed, these reductions were accompanied by an expansion of *A.*

muciniphila and *P. distasonis*, which seems to have a beneficial effect on intestinal epithelial barrier and subsequently in protecting these animals from the development of colitis. *A. muciniphila* is a member of the Verrucomicrobia phylum described in 2004 by Derrien and colleagues [7]. This anaerobe, mucus-degrading bacteria inhabits mainly the mucus layer of the large intestine, representing between 1 up to 4% of the total gut microbiota, and it is ubiquitously distributed in intestinal tracts all over the animal kingdom [8-11]. *P. distasonis* is an anaerobic, Gram-negative bacterium that belongs to the Bacteroidetes phylum [12]. Both *A. muciniphila* and *P. distasonis* have already been described in the context of IBD, but with controversial results. As a matter of fact, *A. muciniphila* is reported to be significantly reduced in biopsies and fecal samples of IBD patients when compared to healthy individuals [13]. On the other hand, *A. muciniphila* can act as a pathobiont in double knockout mice for IL-10 and NLRP6 due to an overgrowth in this bacterium [14]. Similarly, *P. distasonis* is described by some authors as enhancing colitis in mice, while others point its role in attenuating colitis in mice by modulating immunity and microbiota composition [15, 16]. Moreover, *P. distasonis* has been described to be decreased in colon biopsies from patients with CD [17]. The divergent phenotypes described for these bacteria demonstrate that several factors, either environmental or genetic, contribute to the development of IBD. Nevertheless, the fact that both bacteria are found decreased in IBD patients reinforces their putative role in keeping a healthy gut.

The role of these bacteria in other pathologies besides IBD has also been described, although with some discordant reports. As such, decreased abundance of *A. muciniphila* in tissue and fecal samples are also reported in obesity, autism and type 2 diabetes, which is suggestive of a beneficial role of this bacterium in a broad spectrum of diseases [18-21]. This is also extended to anti-tumor therapies, with *A. muciniphila* positively influencing the efficacy of PD-1-based immunotherapy against epithelial tumors [22]. In contrast, *A. muciniphila* was found to be increased in multiple sclerosis patients; interestingly, the same study has observed that *P. distasonis* was reduced in the microbiota composition of those same patients [23].

Indeed, a recent report has demonstrated that *A. muciniphila* and *P. distasonis* can synergistically be involved in the prevention of epilepsy, by reducing gammaglutamylation of amino acids, increasing hippocampal GABA/glutamate ratios and subsequently preventing seizures [24]. This is particularly relevant, since previous studies focusing on the effects of each one of the bacterium in intestinal inflammation, have not addressed the possibility of both acting in combination to induce intestinal protection, which supports the novelty of this work.

Apart from a different microbiota profile, susceptible and resistant mice also display a distinct metabolic signature. Since most identified metabolites can derive from and/or be involved in microbiota metabolism, it is paramount to understand the possible contribution of *A. muciniphila* and *P. distasonis* in metabolite production.

Although no differences were found regarding the main SCFAs, i.e., acetate, propionate and butyrate, the second top hit metabolite that was found to be increased in resistant mice was succinate. Given the increased levels of *P. distasonis* in resistant mice and its capacity to synthesize mostly acetate and succinate [12], we hypothesized that *P. distasonis* could be a major source for the increased levels of succinate present in the intestine of resistant mice. On the other hand, *A. muciniphila* is described as a major propionate and acetate producer mainly through mucin fermentation [7, 25, 26]. Indeed, it was recently described that the production of propionate by *A. muciniphila* can be promoted by vitamin B12 that is used as a cofactor in the conversion of succinate to propionate via methylmalonyl-CoA synthase [27]. Thus, we may infer that a cross-feeding mechanism between *P. distasonis* and *A. muciniphila* can occur in the intestine of resistant mice, with the succinate produced by *P. distasonis* being available for *A. muciniphila* metabolism and colonization.

Besides the succinate pathway, propionate can also be generated by microbial fermentation in the gut through two other pathways: the lactate pathway and the propanediol pathway [28]. Indeed, lactate was the major metabolite found increased in resistant mice and it can probably be derived from species belonging to the *Lactococcus* genus, since this bacterial population was also identified as part of the microbiota of resistant mice. Other metabolites depicted in the protective phenotype, such as leucine and isoleucine, can also contribute to SCFA synthesis, to a lesser extent [29].

The intestinal microbiota is involved in several processes associated with the restoration of homeostasis, such as immunomodulation, production of signaling molecules that target the host or pathogens, and factors that promote increased tight junction barrier function on the epithelia of the host [30]. In the quest to understand how the enrichment in these specific bacteria could be associated with a more protective phenotype, we evaluated the expression of epithelial barrier-associated proteins and intestinal permeability. *A. muciniphila* and *P. distasonis* seem to induce protection by influencing the intestinal epithelial barrier to better tolerate an inflammatory insult. Still, the specific mechanisms underlying this protective phenotype must be dissected. *A. muciniphila* is described to be essential for an healthy mucus layer, promoting mucus production

and thickness [8]. This is reiterated in resistant mice described in our study, which display increased expression of mucin-encoding genes and increased number of goblet cells when compared to susceptible mice, in homeostatic conditions. The increased expression of E-cadherin- and claudin-encoding genes in resistant mice also point towards a modulation of intestinal epithelial barrier to control inflammation and prevent cellular damage. Alterations in the expression of these proteins are widely described in IBD patients, with claudin-2 found to be upregulated, while other claudins, such as claudin-4 and claudin-8, are downregulated [31, 32]. The members of claudin family display different functions. For instance, claudin-1, -3, -4, -5, -7 and -8 confer barrier properties and are found mainly where the epithelium is more tight, such as the distal colon; on the other hand, other claudins such as claudin-2 promote channel formation within the tight junctions and are expressed in leaky epithelia such as the proximal intestine [32]. In resistant mice, we observe an upregulation of claudin-2, -3, -4 and -7, when compared to susceptible mice. This observation was made at homeostatic conditions, where no significant alteration in intestinal permeability was found in either of the groups. Nonetheless, by upregulating diverse claudins, it is suggestive that the epithelial barrier of resistant mice is better prepared to deal with cellular damage and avoid possible translocation of microbial populations from the lumen to the lamina propria.

Similarly, the cytokine profile found in resistant mice is in accordance with a more protected intestinal epithelial barrier, with resistant mice presenting increased levels of IL-10, IL-17 and IL-22 in the colon. IL-10 is essential for intestinal regulation and the assessment of which population may be responsible for the increased amount of this cytokine in resistant mice needs further evaluation. Treg cells are strong candidates for this role, since they are able to respond to bacterial SCFA ligands by expressing GPR43 and not only expand but also increase the production of IL-10 to control proinflammatory responses derived from effector T cells [33]. On the other hand, IL-10 can be ubiquitously produced by immune cells, such as macrophages and dendritic cells, and therefore innate immune cells can also be contributing to the increased amounts of IL-10 found in resistant mice [34]. ILC3 are also highly represented in the intestine of resistant mice, as compared with the susceptible group. These cells are probably responsible for the production of IL-22, but it does not fully explain the increased amounts of IL-17 that are found in the gut of resistant mice. The presence of γδ T cells in the intestine of resistant mice may be a plausible explanation. These cells are well represented in the epithelial sites and not only are IL-17 producers, but also are described to contribute to barrier and tissue repair and

homeostasis [35]. The presence of IL-17 in the intestine is usually associated with intestinal pathology and with an inflammatory environment; nonetheless, the immunotherapy with secukinumab (anti-IL-17A) has shown to be ineffective in IBD patients, suggesting an important regulatory role of this cytokine [36]. Indeed, the impact of IL-17 in the intestine seems to depend on the isoform. The inflammatory responses of IL-17A and IL-17F seem to differ in intestinal disease, with IL-17F deficiency in mice being associated with attenuated DSS-induced colitis, contrary to mice deficient in IL-17A [37]. Similar findings were shown recently by Tang and colleagues, demonstrating that the impact of IL-17 in colitis development is dependent on the isoform, with IL-17F blockade ameliorating colitis, contrary to IL-17A. This observation was explained by an increase in *Clostridium* cluster XIVa in colonic microbiota that is capable of promoting Treg cells [38]. Therefore, IL-17F seems to have a critical role in the regulation of inflammatory responses in the gut. Since we have quantified total IL-17 and not each isoform independently, a further analysis to evaluate both IL-17A and IL-17F is needed to understand if there is a predominance of one of the IL-17 isoforms in the intestine of resistant mice. Finally, IL-22 is known to participate in epithelial repair after injury and expression of antimicrobial peptides [39, 40], suggesting once again that resistant mice display an intestinal epithelial environment more capable of sustaining and overcoming inflammatory stimuli.

Further experiments are needed to confirm that the increased expression of genes encoding for E-cadherin, claudins and mucins, as well as the cytokine profile found in resistant mice, are a direct consequence of *A. muciniphila* and *P. distasonis* presence (alone, or the combination of both proteins). Nevertheless, the preliminary results obtained in the gut-on-a-chip platform suggest that the contact of *A. muciniphila* with the epithelial cells promotes the expression of E-cadherin. The dissection of the mechanisms underlying the impact of these bacterial candidates in intestinal epithelial barrier will be performed in a two-stage approach. Firstly, we are going to supplement susceptible mice with *A. muciniphila* and *P. distasonis* (alone or in combination) and allow the colonization of the bacteria in the gut, followed by the induction of colitis by DSS. The response of these mice to the inflammatory challenge will be evaluated by the disease activity index, in order to assess if the supplementation is sufficient to rescue the protective phenotype and thus validate our candidates as promoters of protection against colitis. Next, we will also unveil the mechanisms by which these bacterial candidates impact the epithelial barrier, by culturing them in the gut-on-a-chip model and assess epithelial barrier integrity, through the evaluation of proteins associated with the regulation of epithelia and

immune response. Since these bacteria may not only have a direct impact on epithelial barrier, but also in the production of metabolites and other mediators (for instance, *A. muciniphila* is described to ameliorate the severity of colitis through the secretion of extracellular vesicles [41]), supernatants from bacteria culturing will also be assessed in the biochip system. Within this integrated approach we aim to unravel the mechanisms by which microbiota can contribute to the regulation of intestinal epithelial barrier and transpose these findings to possible new approaches that promote protection against IBD.

2. PROBIOTICS AND PREBIOTICS AS ADJUVANTS FOR IBD

Due to the lack of an effective therapy for IBD and the advances regarding the impact of colonic microbiota in the outcome of disease development, new possible therapeutic approaches have emerged. Among these, probiotics and prebiotics are in the spotlight for IBD treatment.

Probiotics are mono or mixed cultures of live microorganisms that, when administered in adequate amounts for humans or animals, exert beneficial health effects in the host by restoring the properties of the intestinal microflora [42]. In turn, prebiotics are non-digestible compounds that induce specific alterations in the composition and/or activity of the gastrointestinal microflora, conferring positive effects to the host [43].

Lactobacillus and *Bifidobacterium* species are frequently used as probiotics in IBD management. A clinical trial with UC patients has tested the efficacy of an oral capsule containing *Bifidobacterium* following UC standard therapy, with only 20% of the probiotic group suffering relapse against almost 94% of the placebo group [44]. A recent study has also highlighted the beneficial properties of *Bifidobacterium longum* in intestinal homeostasis, as the administration of these bacteria to mice is able to correct barrier defects by restoring mucus growth [45]. Other reports have demonstrated the positive effects of *Lactobacillus* species in experimental colitis in mice, mostly by decreasing mucosal inflammation [46-48]. Actually, one of the most commonly prescribed probiotics for the induction and maintenance of UC remission is VSL#3, a multispecies probiotic composed by *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Lactobacillus casei*, *Lactobacillus plantarum*, *Streptococcus salivarius* subsp*. thermophilus*, *Bifidobacterium breve*, *Bifidobacterium infantus* and *B. longum* [49]. VSL#3 was demonstrated to attenuate inflammatory responses due to the downregulation of IL-12 and IFN-γ production, reducing neutrophil infiltration and colonic epithelial cell apoptosis. Moreover, it also impacts microbiota composition, promoting the colonization by *Streptococci* and *Lactobacilli* and controlling the expansion of *Enterococcus* species (described to contribute to intestinal inflammation) [49-51].

E. coli Nissle 1917 is described as other probiotic used to aid the treatment of UC since it contributes to the production of anti-inflammatory molecules that act systemically and induces β-defensin production by intestinal epithelial cells, having a beneficial impact in intestinal barrier function via regulation of tight junctions [52]. Clinical studies were performed with *Saccharomyces boulardii* to assess its potential as IBD treatment, with positive results in CD and

UC. Nonetheless, others have shown that *S. boulardii* failed in the treatment of CD, which reinforces the need for further evaluation on the potential clinical usage of this probiotic in IBD management [53-56].

We have identified two distinct bacteria that may have similar protective effects. One of the main challenges in the design of probiotics is the fact that most bacteria found in the intestine are anaerobic and some of them are slow-growers. These are important issues to address, since a proper probiotic needs to assure that the microorganism will be able to survive throughout the gastric cavities until the intestine and also be able to colonize the gut [57]. Although *A. muciniphila* and *P. distasonis* are anaerobic bacteria, it will be interesting to explore in the future the potential probiotic effect of these bacteria and if they can act as prophylaxis or part of the treatment. As previously discussed in this chapter, both *A. muciniphila* and *P. distasonis* produce metabolites, such as SCFAs and other metabolites that can be converted into SCFAs, which are described as beneficial to the intestinal environment, through the downregulation of inflammatory responses and modulation of mucus layer thickness and epithelial barrier integrity. In Chapter II we have observed that susceptible mice that received fecal contents enriched in *A. muciniphila* and *P. distasonis* were able to avoid colitis relapse when challenged with an inflammatory insult, demonstrating that colonization with the given bacteria was successfully achieved. This is particularly relevant since it is known that colitis flares or prolonged antibiotic treatment are associated with a dysregulation of the microbiota composition in the gut [58, 59]. Thus, *A. muciniphila* and *P. distasonis* may emerge as possible candidates for intestinal microbiota reconstitution after an inflammatory event or persistent antibiotic usage, by their administration as prophylactic adjuvant for IBD.

The use of prebiotics within the diet to promote intestinal health has arisen as a potential therapeutic strategy to prevent and aid in the treatment of IBD [60]. The relevance of including fibers in the dietary habits has been widely described [45, 61]. Fibers are fermented by intestinal bacteria into SCFAs, such as acetate, propionate and butyrate, which in turn will impact immune cell activation and epithelial barrier integrity [62-64]. In Chapter III, we have explored the metabolic alterations occurring in the colon of colitic mice. We found a twofold decrease in L-threonine levels during colitis development. Since L-threonine is important for mucin synthesis and previous studies have associated deficits in this amino acid with fewer goblet cells and mucus [65, 66], alongside its decrease during colitis development, L-threonine emerged as a preferential target for supplementation. Thus, we evaluated the supplementation of L-threonine,

at the beginning of colitis and during a well-established inflammatory environment. While supplementation during inflammation had no impact, either beneficial or unfavorable, on disease development and recovery, L-threonine supplementation during colitis onset was found to be detrimental for colitis remission. Indeed, besides a delayed recovery, mice also exhibit fewer goblet cells and reduced amounts of IL-22 in the colon.

Dietary supplementation with L-threonine has already been assessed in the context of colitis, but our findings differ from some of the data available in the literature. For instance, Faure and colleagues [67] used a combination of amino acids (L-threonine, L-proline, L-cysteine and L-serine), not L-threonine singled out, to assess their potential anti-colitogenic effect in rats. The study compared two distinct dietary compositions in terms of amino acid concentration given to rats before and during colitis induction. The authors found that the diet containing the higher dose of amino acids led to an increase in goblet cell number in the epithelial surface of the ulcerated area, stimulated and regulated mucin production in the colon and restored microbiota composition after DSS treatment [67]. The divergent findings in our work can be due to different factors, such as the animal model (rat *versus* mouse), the use of a mixture of amino acids and not L-threonine alone and, importantly, the supplementation period – Faure and colleagues initiated the treatment with the amino acid mixture before colitis induction; instead, we analyzed the impact of L-threonine administration simultaneous to colitis induction or when the disease was already established. Additionally, several studies have pinpointed L-threonine as an essential amino acid for embryonic stem cell renewal and also the regulatory role of L-threonine dehydrogenase in somatic cell reprogramming [68, 69]. This demonstrates that L-threonine can impact metabolic cell remodeling and thus it could be interesting to unveil if this amino acid, besides impacting mucus production and goblet cell synthesis, is acting in specific cell types present in the intestine, from immune to epithelial cells. This may contribute to a better understanding on why L-threonine has a divergent effect during colitis according with the intestinal inflammatory state.

Overall, despite the current advances in probiotic and prebiotic research, further studies are needed to identify how these factors can modulate the intestinal environment, the safety and the dosage required for a beneficial effect, and the design of a time frame of action for their use in IBD therapy.

3. FUTURE PERSPECTIVES IN IBD

Major discoveries in basic research and in clinical settings for IBD have been made in the last years, with a deeper understanding on the genetic variations of the disease, the contribution of microbiome and its immune regulation, and the role of epithelial cells in the maintenance of homeostasis. However, to develop strategies and techniques that are finally successful for the treatment of this disease, there is still the need for an integrated vision of all the components influencing the disease development.

To achieve this, it is paramount to translate more laboratorial findings to the clinics. The establishment of predictive biomarkers of disease outcome is essential to better tailor the treatment of both CD and UC patients. The development of therapies that take advantage of the potential of the gut microbiota, either by promoting the colonization of protective species, or by restoring the balance of microbiota composition with beneficial effects to the host (for instance, through the modulation of dietary composition), should be considered.

Within this work we have explored the versatility of the microbiome in the regulation of intestinal health or disease. We identified two specific bacterial species that seem to induce protection by enhancing epithelial barrier integrity and increasing its tolerance upon external damage. We also pinpointed several metabolites that can be differentially present in the gut during homeostatic or disease contexts. Besides, we took advantage of a groundbreaking complementary *in vitro* model that, by highly mimicking the intestinal environment, is preferential to dissect the exact mechanisms by which the identified bacteria are able to promote protection against colitis. It will also be useful to test and validate the role of specific metabolites in the different cell types present in the intestine. Finally, this biochip-based human gut model can also be used to explore other intestinal pathologies that can be associated with intestinal homeostasis, such as intestinal infection and colorectal cancer, providing new perspectives on the mechanism of action during disease and giving new perspectives for *in vivo* studies and, subsequently, to novel clinical strategies.

In recent years, several studies have associated alterations in the intestinal microbiota composition with several pathologies, including IBD [70-73]. Nevertheless, bacterial profiles in the gut can rapidly fluctuate due to environmental cues, and this represents a huge challenge in the identification of specific beneficial microbes to intestinal health. Knowing this, we envisage the development of an observational longitudinal prospective study, aiming to associate the

intestinal microbiota composition with relapse-remission events inherent to the disease. In particular, it will be of major interest to dissect the possible association between the microbial composition in the gut, including *A. muciniphila* and *P. distasonis*, with disease severity and intestinal barrier function. By pinpointing the gut microbiota fluctuations during disease and, in parallel, exploring the effect of the identified bacteria in intestinal function (for instance, in the gut-on-a-chip model), this strategy can contribute to the design of new personalized therapies with the core purpose of a long-lasting or, at least, more effective remission of the disease.

Overall, there are still several questions to be answered in the context of IBD. The identification of the ideal biomarkers for patient stratification regarding disease course or response to treatment would be extremely valuable. Additionally, the design of optimized prophylactic and treatment strategies, either by immunomodulators, probiotics/prebiotics or the combination of both, and subsequent adaption to the disease profile of patients is urgent, taking into consideration the increased number of IBD cases. In this sense, microbiome-derived therapies seem to be a reality for IBD prophylaxis and treatment, and despite the need to evaluate if microbiota modulation can lead to long-term effect on disease remission, it is undoubtedly an attractive field to explore in order to design new strategies of tackling IBD.

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