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ROLE OF BACTERIA AND THE MUCUS SYSTEM IN INTESTINAL TUMORIGENESIS

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ABBREVIATIONS

AB	Alcian blue
AhR	Aryl hydrocarbon receptor
AID	Activation-induced deaminase
AJ	Adherens junction
ALT	Alanine transaminase
AMP	Antimicrobial peptide
AOM	Azoxymethane
APC	Adenomatous polyposis coli
ASF	Altered Schaaedler flora
Bp	Base pairs
BSA	Bovine serum albumine
C	Carboxy
CCR	Chemokine receptor
CD	Cluster of differentiation
CD	Crohn's disease
CFU	Colony forming unit
CRC	Colorectal cancer
CSR	Class switch recombination
CTLA4	Cytotoxic T-lymphocyte antigen 4
Cx	Connexin
CX3CR1	Chemokine (C-X3-C motif) receptor 1
DC	Dendritic cell
DEF	Defensin
DNA	Deoxyribonucleic acid
DSS	Dextran sodium sulphate
EC	Epithelial cell
FAP	Familial adenomatous polyposis
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FDC	Follicular dendritic cell
FXR	Farnesoid X receptor
GAP	Goblet cell-associated passage
GC	Germinal centre
GF	Germ free
GI	Gastro-intestinal
GITR	Glucocorticoid induced TNF receptor family related gene
GJ	Gap junctions

GPBAR1	G protein-coupled bile acid receptor 1
GSK3β	Glycogen synthse kinase 3β
hCRC	Human colorectal cancer
HID	High iron diamine
HNPCC	Hereditary non-polyposis colorectal cancer
Hpi	Hours post infection
HRP	Horseradish peroxidase
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cell
IEL	Intra-epithelial lymphocyte
Ig	Immunoglobulin
IL	Interleukin
ILF	Isolated lymphoid follicle
iTregs	Induced Tregs
J	Joining
LPS	Lipopolysaccharide
LOH	Loss of heterozygosity
M cell	Microfold cell
MALT	Mucosal-associated lymphoid tissue
MAMP	Microbe-associated molecular pattern
MDSC	Myeloid derived suppressor cell
MHCII	Major histocompatibility complex class II
Min	Multiple intestinal neoplasia
miRNA	MicroRNA
mLNs	Mesenteric lymph nodes
MMP	Matrix metalloproteinase
Muc	Mucin
N	Amino
N	Nitrogen
NAC	N-acetyl cysteine
NF-kB	Nuclear factor kappa B
NK	Natural killer
NLRs	nucleotide-binding oligomerization domain (NOD)-like receptors
NOD	Nucleotide-binding oligomerization domain-containing protein
nTregs	Natural Tregs
O	Oxygen
OTU	Operational taxonomic unit
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PGN	Peptidoglycan
pIgR	Polymeric Ig receptor
PMA	Phorbol myristic acid
PP	Peyer's Patch
PRR	Pattern recognition receptor
RA	Retinoic acid
REG	Regenerating islet-derived protein
RNA	Ribonucleic acid
ROS	Reactive oxygen species
Rpm	Revolutions per minute
qPCR	Quantitative polymerase chain reaction
RT	Retro-transcription
SC	Secretory component
SEM	Standard error mean
SHM	Somatic hypermutation
SI	Small intestine
sIgA	secretory IgA
SAA	Serum amyloid A
SFB	Segmented filamentous bacteria
STAT	Signal transducer and activator of transcription
TAM	Tumor-associated macrophage
TAN	Tumor-associated neutrophil
TCR	T cell receptor
Tfh	T follicular helper
Tfr	T follicular regulatory
TG	Transgenic
TGF-β	Transforming growth factor β
Th	T helper
Th17	T helper 17
TJ	Tight junction
TLR	Toll-like receptor
TMB	Tetramethylbenzidine
TNF-α	Tumor necrosis factor α
Tregs	T regulatory cells
TSLP	Thymic stromal lymphopoietin
UC	Ulcerative colitis
UTR	Untranslated region
VEGF	Vascular endothelial growth factor

WT	Wild type
ZO-1	Zonula occludens 1

ABSTRACT

Many studies on human colorectal cancer (hCRC) samples have documented a dysbiosis associated with the tumor at different levels. However a clear picture of the microorganisms whose abundance is altered during tumorigenesis and evidences of their functional implication in the tumorigenic process have not yet emerged.

This study is aimed at dissecting the role of bacteria in CRC development by focusing on the intestinal mucus barrier as a key mediator in microbiota-host interaction.

We used the $Apc^{Min/+}$ murine model to demonstrate that in tumor-bearing mice, similarly to what happens in hCRC, the mucus barrier has altered properties. Mucin expression is altered at the level of dysplastic crypts, strictly relating mucus changes with neoplastic transformation. Being the mucus a fundamental component of the intestinal barrier we further addressed if bacterial penetrance was compromised in tumor-prone mice. As hypothesised upon oral administration we observe increased *Salmonella* penetration in the intestine and spreading to mesenteric lymph nodes of $Apc^{Min/+}$ mice compared to wild types.

To address the potential role of mucus alteration in the tumorigenic process we exogenously modified the mucus barrier. Upon treatment with *Salmonella*, a bacterium that is able to interact with the mucus, we observed an increase in colon tumorigenesis in $Apc^{Min/+}$ mice. Moreover, when we coupled the AOM/DSS protocol of chemically induced colitis-associated colon carcinogenesis to mucolytic treatment we observed exacerbated colon tumorigenesis.

We did also investigate whether there was a dysbiosis associated with tumor progression, and when it was arising. Metagenomic analysis in the faeces of mice at different ages highlighted a dysbiosis already at 4 weeks of age in $Apc^{Min/+}$ mice when tumors are not yet established. In particular, *Lactobacillus* genus was expanded in $Apc^{Min/+}$ mice concomitantly with a contraction in the *Clostridium* genus. At the species level *Clostridium* ID4 was underrepresented in $Apc^{Min/+}$ mice both in the faeces and at the level of mucus-associated bacterial community. Exogenous administration of *C*. ID4

to $Apc^{Min/+}$ mice did result in neither any major change in tumor multiplicity nor alteration in gut resident immune cell populations.

Finally since epigenetic mechanisms have recently been hypothesised to contribute to the loss of heterozygosity of the normal *apc* allele and bacteria can alter miRNA expression we analysed miRNA profiles in WT and $Apc^{Min/+}$ intestinal tissue observing alterations in $Apc^{Min/+}$ ilei. We further tested if bacterial stimulation could drive alterations in the *apc* gene expression, possibly mediated by miRNA modulation, in the intestinal mucosa. *Salmonella* and *E. coli*, either invasive or not, did not produce alterations in *apc* gene expression in an *ex-vivo* organ culture model that allows polarized stimulation of the intestinal mucosa.

In conclusion we found that the mucus layer and intestinal barrier properties are altered in tumor bearing mice. This could lead to the establishment of a dysbiosis, although the cause-effect relationships of this do not emerge clearly from the present work. Bacterial species that are underrepresented in tumor bearing subjects could be protective and their administration could delay tumor progression. Finally, dysbiosis could induce miRNA-mediated control of *apc* gene expression and this could be a mechanism involved in tumor initiation in the model used. However, even if bacterial stimuli can modulate miRNA expression patterns, the ones tested in this work are not efficient in modulating *apc* expression.

1 INTRODUCTION

The epithelial surfaces of the human body are continuously challenged by harmful environmental agents and their integrity is fundamental in homeostasis maintenance.

Through the gastrointestinal (GI) tract we introduce every day food antigens and microbes our organism has to cope with. Moreover, the GI tract is the home niche for the intestinal microbiota with which a symbiontic relationship is established. To interact with all these agents the organism has evolved a multi-layer barrier that has on one side the role of protecting from pathogens and harmful agents and on the other side of allowing and promoting tolerance and exchange with the commensal microflora (Hooper and Macpherson, 2010). The endogenous microbiota has a fundamental role in maintaining host homeostasis and alterations of the microbal community are usually paralleled by disease status (Eberl, 2010; Rescigno, 2008).

Colorectal cancer (CRC) is a leading cause of cancer-related death and being the intestine a protagonist in the mutualism with the gut microbiota lots of effort has been made to elucidate its possible role in CRC pathogenesis (Irrazabal et al., 2014).

This introduction will describe the principles of the intestinal barrier, the intestinal microbiota and colorectal cancer, highlighting the interactions among these elements.

1.1 The intestinal barrier

A mucus layer whose characteristics vary along the different GI segments is the first level of defense of the organism towards bacteria and fed antigens. Moreover the GI tract is lined by a specialized epithelium containing cells that harbor peculiar functions, such as Goblet cells and Paneth cells (Hooper and Macpherson, 2010). Finally, the intestinal lamina propria is patrolled by a number of immune cell populations such as IgA-secreting plasmacells, dendritic cells (DCs), T regulatory cells (Tregs) and T helper 17 (Th17) cells (Hooper and Macpherson, 2010). All of these elements together form

the intestinal barrier shown in Figure 1-1 and its components will be described in the following paragraphs.

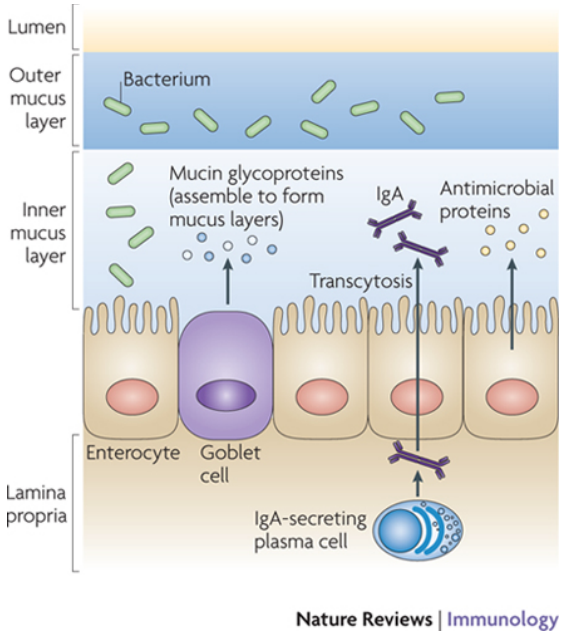

Figure 1-1 The intestinal barrier. The intestinal barrier is composed by a mucus layer overtopping the intestinal epithelium. Antimicrobial peptides and secretory IgAs are secreted in the gut lumen while the lamina propria is patrolled by immune cells. Adapted from (Hooper and Macpherson, 2010).

1.1.1 The mucus barrier

The mucus barrier is a fundamental component of the intestinal barrier that has only in recent years attracted the attention it actually deserves. The mucus is the very first interface between the host and microbes, both commensals and pathogens, and it has the dual role of being the home niche to commensal microorganisms and to protect the host from them and from pathogens (McGuckin et al., 2011).

The mucus is a characteristic of the entire GI tract, but is composition and thickness vary in the different tracts according to the different local microenvironment (Figure 1-2). For example in the stomach there is very thick mucus layer (up to 700 μm) that is fundamental in protecting the gastric epithelium from the acidic pH of the stomach while in the intestine, where absorptive processes do take place, it is thinner (150 to 300 μm). Also within the intestine there are substantial differences between the small intestine (SI)

where the mucus is patchy and not continuous and the colon where it keeps physically apart the commensal microbiota, reaching in this tract its maximal density (McGuckin et al., 2011).

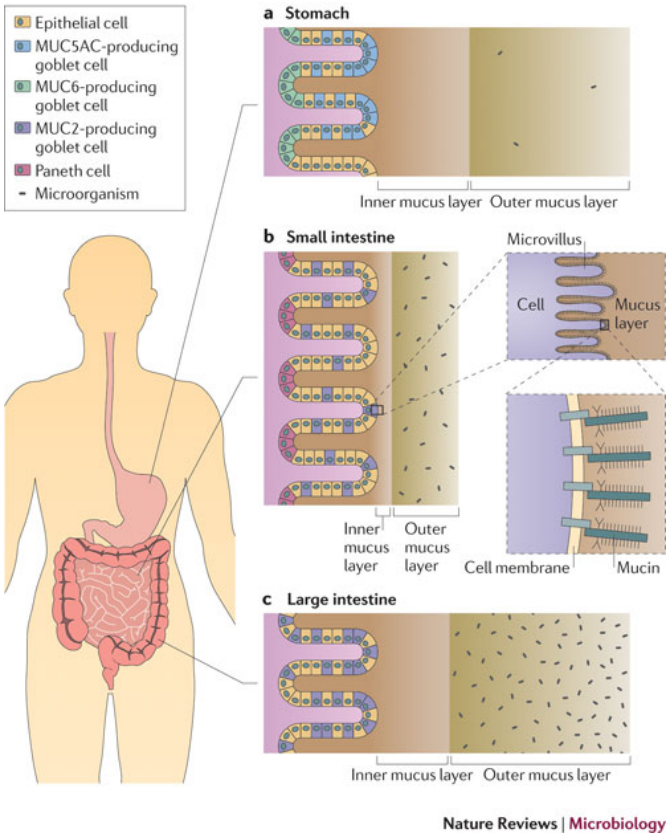

Figure 1-2 The mucus barrier. The GI tract is characterized by the presence of a mucus layer whose characteristics and thickness vary among the different segments. Adapted from (McGuckin et al., 2011).

1.1.1.1 Mucins

The mucus is formed by high molecular weight filamentous glycoproteins that are produced by specialized secretory cells called Goblet cells. Mucins are synthesised in the endoplasmic reticulum and undergo heavy nitrogen (N)- and oxygen (O)-linked glycosilations. Mucins can be either secreted or membrane bound and they form an highly hydrated net in the extracellular space (McGuckin et al., 2011).

Secreted mucins are heavily glycosylated proteins that confer the mucus its gel-like properties. Mucin (MUC) 2, MUC5AC, MUC5B, MUC6 and MUC19 form homooligomers through disulfide bonds occurring at the amino (N)- and carboxy (C)-terminal domains,

while MUC7 is a non oligomerizing secreted mucin. An important role of the secreted mucins is to form a gel that acting like a sponge retains antimicrobial factors secreted by the host, that would otherwise be lost in the luminal space. The mucus retains important molecules such as antimicrobial peptides (e.g. defensins) and secretory (s) IgAs at high concentrations in proximity to the epithelium (McGuckin et al., 2011; Ouwerkerk et al., 2013). In addition to this mucins can act themselves as antimicrobial determinants as has been demonstrated for pyloric gland-secreted mucus, whose terminal monosaccharide α(1,4)-linked *N*-acetylglucosamine inhibits the synthesis of fundamental components of *Helicobacter pylori* cell wall (Kawakubo et al., 2004). Moreover Muc5ac is upregulated in the caecum of mice infected with the nematode *Trichuris muris* and it is involved in direct reduction of nematode viability and in the establishment of a proper IL13-driven Th2 response fundamental for the resolution of the infection (Hasnain et al., 2011).

Membrane-bound mucins are an important component of intestinal epithelial cells (IECs) glycocalix. Membrane-bound mucins expressed in the GI tract include MUC1, MUC3A, MUC3B, MUC4, MUC12, MUC13, MUC15, MUC16 and MUC17. During their biosynthesis membrane bound mucins are cleaved and subsequently exposed on the plasma membrane as two non-covalently linked subunits that interact through a SEA module. Disruption of this interaction leads to the shedding of the extracellular mucin domain and is both a naturally occurring process and a mechanism of pathogen clearance (Linden et al., 2008; Sheng et al., 2012). Membrane bound mucins are the first elements pathogen that have managed to reach the epithelial layer come in contact with. MUC1 mucin, prevalently expressed in the stomach, has a pivotal role in preventing *Helicobacter pylori* infection. Most *H. pylori* strains express adhesins that bind to gastric cell-bound MUC1. MUC1 acts as a decoy receptor that is shed upon bacterial binding leading to the release of MUC1 coated bacteria. Moreover, MUC1 prevents invasion by *H. pylori* strains that do not express adhesins by simple steric hindrance (Linden et al., 2009). MUC1 mucin is upregulated in mice upon *Citrobacter rodentium* infection and in humans infected with *Salmonella* St Paul, *Salmonella*

enteridis, *Campylobacter jejuni* and *Clostridium difficile* (Linden et al., 2008). However contrarily to expectations, *Muc1*$^{-/-}$ mice are protected from dextran sodium sulphate (DSS)-induced colitis. This is explained by increased mucus thickness in these mice, compensatory increase in Muc3 expression and reduced recruitment of infiltrating T lymphocytes (Petersson et al., 2011). Mice deficient in the membrane bound mucin MUC13 are more susceptible to DSS-induced colitis as a result of increased apoptosis and reduced proliferation of colonic epithelial cells, showing a protective role for this mucin during inflammation (Sheng et al., 2011).

Bacteria can bind mucins and establish their niche in the mucus, degrading it and using it as an energy source (Ouwerkerk et al., 2013). *Akkermansia muciniphila* is an example of bacterial species that express mucus-degrading enzymes. This species has been isolated from the human microbiota and is associated with health host status while depleted in IBD (Ouwerkerk et al., 2013). Moreover mucus components can act as anchoring sites for pathogens that can subsequently invade the host. *S.* typhimurium binding to mucus glycoproteins occurs *in vivo* and is important for invasion (McCormick et al., 1988; Nevola et al., 1987; Vimal et al., 2000).

Membrane-bound mucins also have intracellular domains that take part in signalling processes. As an example of this the intracellular C-terminal domain of MUC1 can be bound and phosphorylated by glycogen-synthase kinase (GSK)-3β disrupting its binding to β-catenin. The free β-catenin localizes to E-cadherin adherens junctions rather than affecting β-catenin dependant transcriptional activity (Li et al., 1998). Overexpression of MUC1, on the contrary, recruits β-catenin from adherens junctions (Li et al., 1998). Moreover both MUC1 and MUC13 are involved in the NF-κB mediated inflammatory response triggered by tumor necrosis factor (TNF)-α and TLR and NOD ligands even though with opposing effects. In fact MUC1 and MUC13 act respectively decreasing and increasing IkBα phosphorylation and degradation, thus decreasing and increasing NF-kβ p65 translocation to the nucleus and the subsequent inflammatory response. The meaning of this opposite effect in the context of intestinal homeostasis could be that when MUC13, normally expressed by IECs, comes in contact with microbes that have

breached the outer lines of defense it triggers inflammation together with PRRs. On the contrary MUC1 is upregulated upon infection and acts as a "sheddin" preventing bacterial binding to epithelial cell surface and shutting down the inflammatory response that would otherwise become detrimental for the organism (Sheng et al., 2013).

In the colon the mucus has a peculiar organization with an inner dense and firm layer, closely attached to the epithelium, and an outer loose layer (Figure 1-3). This structural organization has an important functional meaning since bacteria colonize exclusively the outer layer (Johansson et al., 2008). The main component of intestinal mucus is the secreted gel-forming mucin MUC2. The inner firm layer is continuously regenerated by Goblet cells, while the outer loose layer is generated from the inner one in a process that involves proteolytic cleavage of MUC2 (Johansson et al., 2008).

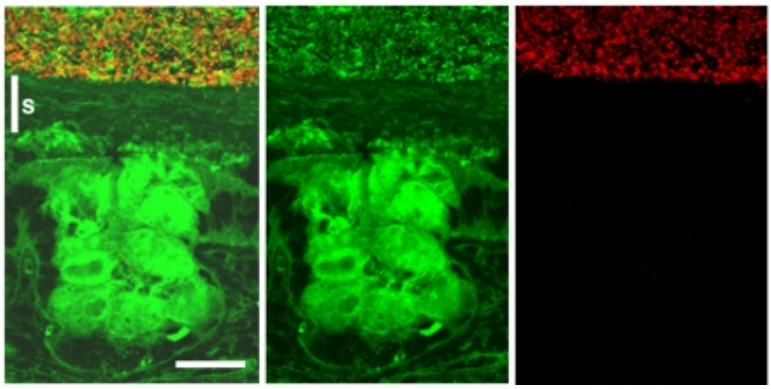

Figure 1-3 The mucus is composed by an inner firmly attached and an outer loose layer. The inner layer is almost sterile while the outer layer is heavily colonized by bacteria. Immunofluorescence shows MUC2 (green) and 16S bacterial DNA (red). Adapted from (Johansson et al., 2008).

Further evidence of the fundamental role of the mucus layer in homeostasis comes from *Muc2*$^{-/-}$ mice that do spontaneously develop colitis and CRC (Velcich et al., 2002). *Muc2*$^{-/-}$ mice are also more susceptible to *Citrobacter rodentium* infection (Bergstrom et al., 2010) and upon DSS challenge they display worst phenotype compared to wild type (WT) mice and translocation of anaerobic bacteria to the spleen (Petersson et al., 2011). Mucins are either constitutively expressed or induced upon specific microbial and immune signals. Many probiotics increase mucin expression, thus impeding pathogen

entry, while some pathogens use mucin downregulation as an invasion mechanism. For example *H. pylori* infection is associated with reduced gastric expression of MUC5AC and MUC6 and the bacterium produces enzymes able to degrade sulphated mucins (Sheng et al., 2012). Moreover many proinflammatory cytokines induce mucin expression and secretion both in goblet cells and in intestinal epithelial cells (Sheng et al., 2012).

1.1.1.2 The mucus in oral tolerance

Recent works have highlighted that the mucus does not form a simple physical barrier in the intestine but rather has a role in antigen sampling and establishment of oral tolerance, the state of local and systemic immune "ignorance" towards commensal microbes and food antigens. McDole and colleagues have shown with intra-vital microscopy experiments that Goblet cells are taking up antigens from the intestinal lumen and interact with tolerogenic CD103+ DCs on the basolateral side (McDole et al., 2012). The formation of these Goblet cell-associated passages (GAPs) is regulated by acetylcholine in the small intestine, while in the colon it is tightly regulated through MyD88 signalling (Knoop et al., 2014). Disruption of microbial sensing results in colonic GAPs formation and antigen delivery to colonic antigen presenting cells (APCs) (Knoop et al., 2014). Additional work has shown that the uptake of MUC2 coated bacteria confers a tolerogenic phenotype to DCs, characterized by reduced expression of proinflammatory cytokines and increased production of TGF-β, IL-10 and RA metabolizing enzymes. This tolerogenic microenvironment favours the differentiation of Treg cells. SI DCs bind MUC2 glycosidic residues via Galectin3, Dectin1 and FcγRIIB and acquire MUC2 on their surface from SI ECs. Dectin1 signalling mediates AKT and GSK-3β phosphorylation, promoting the stabilization and nuclear translocation of β-catenin. Nuclear translocation of β-catenin disrupts NF-κB p50-p65 binding avoiding transcription of proinflammatory genes (Shan et al., 2013).

1.1.1.3 The mucus barrier in disease

A number of polymorphisms in mucin genes are associated with inflammatory bowel disease (IBD). As a matter of fact polymorphisms in MUC3 and MUC2 genes are associated with Crohn's disease (CD) while polymorphisms involving MUC4 and MUC13 with ulcerative colitis (UC) (Sheng et al., 2012). In addition to mutational alteration of mucin genes mucus alterations can arise as a result of aberrant expression, maturation or secretion of mucin genes.

The effect of deficiencies in many mucin genes has been investigated in animal models that demonstrate their fundamental role in maintenance of intestinal homeostasis, as previously discussed in paragraph 1.1.1.1. The expression level of some mucins is reduced in IBD, while MUC5AC, which is not normally expressed in the healthy intestine, is frequently expressed in both UC and CD (Sheng et al., 2012). Mucin glucidic patterns are often altered in IBD leading to incomplete or less complex glycosylation. Mice genetically engineered to lack core O-glycans or to have reduced sulphonation activity are generally prone to intestinal inflammation, more susceptible to DSS-induced colitis and in the first case do also develop CRC (Sheng et al., 2012).

Finally it has been demonstrated that the lack of functionality of the NLRP6 inflammasome causes an impaired mucus secretion due to aberrant autophagy pathway in goblet cells (Wlodarska et al., 2014). The lack of a properly assembled mucus layer favours the establishment of a colitogenic microbiota that is transmissible to WT animals (Elinav et al., 2011) underlining the importance of host factors in shaping the intestinal microbial community that is in turn able to transfer a dominant phenotype to an healthy host.

Mucus alterations occurring in CRC will be discussed in paragraph 1.3.2

1.1.2 The intestinal epithelium

The intestinal mucosal surface is lined by a monolayer of columnar epithelial cells (ECs). IECs are polarized and are in contact at the apical side with the intestinal lumen and at the basolateral side with lamina propria resident immune cells. The apical side of IECs

is organized in a brush border to favour absorption of water and nutrients from the diet (Brown et al., 2013; Hooper and Macpherson, 2010; Iliev et al., 2007). Moreover IECs are sealed to each other through adherens junctions (AJs) and tight junctions (TJs) formed by specialized proteins from the claudin and occludin families preventing bacteria to pass in the lamina propria and allowing tight control of pericellular passage of molecules (Brown et al., 2013; Hooper and Macpherson, 2010; Iliev et al., 2007).

The intestinal epithelium does also contain many specialized cell types.

Microfold cells (M cells) are important components of the follicle-associated epithelium of the Peyer's Patches (PPs), organized secondary lymphoid structures of the mucosal-associated lymphoid tissue (MALT). These cells are specialized in transcytosis and take-up antigens from the intestinal lumen and pass them to immune cell populations of the sub-epithelial dome, such ad B and T lymphocytes, macrophages and DCs (Jang et al., 2004).

Other specialized cell types comprise Goblet cells and Paneth cells, whose functions are described in paragraphs 1.1.1 and 1.1.3 respectively.

But the epithelial barrier is not a mere physical barrier to food and microbes. Recent work has highlighted its active role in sensing the microbial community of the intestine (Brown et al., 2013; Iliev et al., 2007). In fact IECs express pattern recognition receptors (PRRs), proteins specialized in the recognition of microbe-associated molecular patterns (MAMPs), conserved structures shared among different classes of microbes, both pathogens and not (Brown et al., 2013; Iliev et al., 2007). PRRs include Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (Brown et al., 2013; Iliev et al., 2007). PRRs sense structures shared among the microbial world, such as peptidoglycan (PGN), lipopolysaccharide (LPS), flagellin, dsRNA, ssRNA and CpG islands (Brown et al., 2013). The ability of PRRs to discriminate between commensals and invading pathogens is due to their compartmentalization (Iliev et al., 2007). In fact some TLRs are expressed only at the basolateral membrane of IECs, thus allowing exclusive response towards invading microbes. An example for this is TLR5 that is expressed on the basolateral membrane

of IECs and on DCs. In this way it is not activated at steady state, but when its ligand flagellin invades the lamina propria it participates in the induction of innate responses (Eberl, 2010; Iliev et al., 2007). NLRs are intracellular receptors that can sense ligands secreted by pathogens that express type III or type IV secretion system, such as *Salmonella enterica* serovar typhimurium and *Helicobacter pylori* (Iliev et al., 2007). Within this framework MAMPs do not define pathogens but rather pathogenic situations in which any microbe could be involved: virtually any microbe can become a pathogen depending on the context and on the mutualism with the host (Eberl, 2010).

Proper sensing of the microbial environment by IECs is fundamental for intestinal homeostasis. Nuclear factor (NF)-κB mediated signaling in IECs is essential in driving proper responses towards pathogens. Mice deficient for IκB kinase (IKK)-β fail in the clearance of the intestinal parasite *Trichuris muris* and develop a detrimental Th1- and Th17-dominated response rather than a pathogen-specific Th2 response that is fundamental in infection clearance (Zaph et al., 2007).

Finally, IECs can secrete cytokines that play important roles in the differentiation of immune cell populations in the lamina propria. Accordingly, epithelial cell-derived transforming growth factor-β (TGF-β), retinoic acid (RA) and thymic stromal lymphopoietin (TSLP) promote the differentiation of human immature DCs into tolerogenic CD103+ DCs. These DCs are fundamental in maintaining homeostasis with the commensal microbiota and promoting the generation of functional Tregs (Iliev et al., 2009b). In the murine system TSLP is dispensable in this process (Iliev et al., 2009a).

1.1.3 Antimicrobial peptides

Antimicrobial peptides (AMPs) are small molecules produced and secreted by Paneth cells. The mucus layer entraps AMPs acting like a sponge and keeps their concentration higher near the epithelium where they can exert their function and cooperate in keeping microorganisms away from the intestinal epithelium (Brown et al., 2013; Hooper and Macpherson, 2010). AMPs, that include defensins and C-type lectins, have different mechanisms of action and this allows efficacy towards a wide range of microorganisms

(Brown et al., 2013; Hooper and Macpherson, 2010). For example, most defensins are cationic peptides that disrupt Gram-negative outer membrane forming pore-like structures, while the C-type lectin regenerating islet-derived protein (REG) III-γ prevents Gram-positive bacteria from binding IECs interacting with bacterial PGN. However while many AMPs are microbicidal others, such as human α-defensin (DEF)-6, bind to the bacterial surface by forming nets that cause bacterial aggregation and block bacterial entry. Some defensins are constitutively expressed while others are induced upon microbial sensing (Brown et al., 2013).

Studies in murine models have highlighted the fundamental role of AMPs in maintenance of homeostasis with the intestinal commensal community. Transgenic (TG) mice overexpressing human defensin DEFA5 have altered small intestinal microbiota in terms of composition but not total number of bacteria. In particular these mice have a reduction in Firmicutes and an increase in Bacteroidetes. Mice lacking the enzyme matrix metalloproteinase (MMP)-7, whose proteolytic cleavage is required for murine α-defensins activation, have on the contrary an increase in Firmicutes and a decrease in Bacteroidetes (Salzman et al., 2010). The species *Candidatus arthromitus*, belonging to the Clostridiales group and better known as segmented filamentous bacteria (SFB), is in close contact with IECs in WT mice while it is not present in DEF5A-TG mice, underlining the fundamental role of this defensin in tuning the abundance of bacterial species in close relationship with the host. DEFA5-dependant decrease in SFB correlated with decreased abundance of lamina propria CD4+ IL17 expressing T cells. A reduction of α-defensin expression is documented in Crohn's disease. These alterations could lead to dysbiosis that in turn perpetuates inflammation (Salzman et al., 2010).

Degranulation of Paneth cells to secrete AMPs can be induced by different stimuli, such as neurotransmitters activating muscarinergic acetylcholine receptors or bacterial determinants. Studies performed on SI organoids have demonstrated that even if Paneth cells do express PRRs they do not degranulate upon MAMPs stimulation. However, stimulation with IFN-γ at physiologically relevant concentration causes degranulation and secretion of lysozyme and defensins in the organoid lumen (Farin et

al., 2014). Prolonged stimulation with IFN-γ results in Paneth cells extrusion in the crypt lumen. The extruded nuclear deoxyribonucleic acid (DNA) could act as a microbe entrapping net similarly to what happens with activated neutrophils. Natural killer (NK) T and CD3 cells activated upon microbial sensing represent the physiological source of IFN-γ. The unresponsiveness of Paneth cells to direct microbial stimulation could be aimed at avoiding continuous stimulation by commensals in the gut lumen and guarantee degranulation only upon invasion (Farin et al., 2014).

Moreover, mice deficient in the antimicrobial protein REGIII-γ display alterations in mucus distribution and consequent closer bacteria-epithelium interactions in the small intestine, resulting in low grade inflammation elicited by innate immune cell infiltrates and IL22 (Loonen et al., 2014).

1.1.4 Secretory IgAs

IgAs are the most abundant antibodies present in mucosal secretions. IgAs are produced in the intestinal lamina propria by IgA-secreting plasmacells and interact with the joining (J) chain forming dimers. Dimeric IgAs bind to the polymeric Ig receprtor (pIgR) on the basolateral membrane of IECs that mediates secretion in the gut lumen through transcytosis. sIgA are stabilized by the secretory component (SC).

IgA producing plasmacells differentiate in the germinal centres (GCs) of PPs and isolated lymphoid follicles (ILFs) where DCs, follicular dendritic cells (FDCs) and T follicular helper (Tfh) cells produce anti-inflamamtory mediators, such as TGF-β1. In this context plasmacells undergo class switch recombination (CSR) to IgA and somatic hypermutation (SHM) to generate the IgA repertoire. IgA producing plasmacells then home to the lamina propria through the chemokine receptor (CCR) 9 and the integrin α4β7 and here they produce IgAs that are then secreted in the intestinal lumen (Gutzeit et al., 2014).

IgA induction can be induced also by T cell independent pathways occurring at both follicular and extrafollicular sites (Gutzeit et al., 2014). Intestinal DCs can in fact present antigen directly to B cells in the presence of IL6, RA, TGF-β and IL10. Moreover DCs

can release BAFF and APRIL that are involved in IgA CSR and IECs by producing TSLP can stimulate DC expression of these two mediators in a feed forward loop that favours IgA production in a T cell independent manner (Gutzeit et al., 2014).

The small intestinal IgA repertoire is more polyclonal than previously thought, with a few highly expanded clones and many low frequency clones. There is high inter-individual variability in the IgA repertoire and ageing increases diversity. Interestingly highly expanded clones are present in young mice, meaning that low frequency clones might originate from the previous ones through SHM. IgA diversification depends on microbiota, T cells and secondary lymphoid structures since germ-free, $CD3^{-/-}$ and *Rorγt*$^{-/-}$ mice have reduced diversity in the IgA repertoire. Moreover the IgA repertoire before and after plasmacell depletion is very similar, suggesting the existence of memory cells that are recruited from the periphery to the intestine (Lindner et al., 2012).

IgA have a fundamental role in maintaining the balance with the commensal community avoiding pathogen entry but also in shaping the microbial community of the intestine. Activation-induced cytidine deaminase (AID) is an enzyme involved in CSR and SHM and mice lacking this enzyme do not have IgA-producing plasmacells. Sequencing of bacterial 16S ribosomal gene highlights a dysbiosis in $AID^{-/-}$ mice with aberrant expansion of the anaerobic compartment. SFB, that normally colonizes the lower small intestine in mice, is abundant in the upper SI of IgA deficient mice but is re-confined to the ileum upon IgA reconstitution (Suzuki et al., 2004). Moreover it has recently been shown that FoxP3+ T cells differentiate in T follicular regulatory (Tfr) cells in the GCs and promote IgA production. This comes as part of a feed-forward loop where a rich microbiota sustains FoxP3+ T cell mediated IgA production that in turn controls the diversification of the microbiota pointing out how the adaptive immune response acts to favour establishment and maintenance of a diversified microbiota (Kawamoto et al., 2014).

1.1.5 Dendritic cells and macrophages

Dendritic cells are fundamental players in the maintenance of oral tolerance. The two principal subtypes of phagocytes patrolling the intestinal lamina propria are the tissue resident macrophage like chemokine (C-X3-C motif) receptor 1 (CX3CR1) + macrophages and the migratory CD103+ DCs. Phagocytes in the small intestine show constitutive expression of TJ proteins such as β-catenin, occluding and claudin-1, while zonula occludens (ZO) 1 is induced upon bacterial stimulation. This allows extrusion of dendrites through opening of the TJs that seal the intestinal epithelium without compromising its barrier function. In this way DCs directly sample luminal antigens (Rescigno et al., 2001). Recently Mazzini and colleagues have shown that the different antigen presenting cells subsets present in the lamina propria cooperate in the establishment of tolerance. Fed antigen sampling is mainly operated by CX3CR1+ macrophages that then transfer it through physical contact to CD103+ DCs. Antigen transfer takes place through gap junctions (GJ) formed by connexin (Cx) 43 hemichannels. After antigen acquisition CD103+ DCs migrate to mLNs where they promote Tregs differentiation and the establishment of tolerance (Mazzini et al., 2014).

1.1.6 Th1 and Th2 cells

Naïve T cells can differentiate into different types of effector cells and this fate is guided by the cytokine microenvironment determined by the innate arm of immunity.

CD4 expressing T cells can differentiate in different T helper (Th) cell types each one characterized by specefic cytokine profiles and thus effector functions. The first Th subsets to be characterized were Th1 and Th2 subsets.

Naïve T cells differentiate in Th1 cells upon T cell receptor (TCR) activation in concomitant exposure to IFN- γ and IL-12 produced by macrophages, DCs and NK cells upon infection. This causes signal transducer and activator of transcription (STAT) 1 mediated upregulation of T-bet, the master transcription factor of the Th1 cell subset. Cells expressing high levels of T-bet upregulate IL-12Rβ2 and IL-18Rα and become

sensitive to IL-12 and IL18. These mechanisms cooperate in stimulating the production of IFN-γ that leads to macrophage and other immune cell types activation and IgG2a production by B cells, aimed at the clearance of intracellular pathogens (Weaver et al., 2007; Zhu and Paul, 2008).

The Th2 differentiation program is triggered upon TCR stimulation together with STAT6 activation mediated by IL4 produced by basophils, eosinophils, mast cells and NKT cells. STAT6 upregulates the Th2 master transcription factor GATA3 that induces production of IL4, IL5 and IL13. Th2 cells condition B cells to produce IgG1 and IgE. Th2 cells are important in the clearance from extracellular pathogens and helmints (Weaver et al., 2007; Zhu and Paul, 2008). TSLP-conditioned DCs or TSLP cytokine alone can prime naïve T cells towards a Th2-like phenotype with prominent IL4, IL5 and IL13 production (Soumelis et al., 2002; Spadoni et al., 2012).

Th1 and Th2 responses are finely tuned and an exacerbated response leads to autoimmune disease in the first case and allergy in the second.

1.1.7 Th17 cells

Th17 cells are another T helper subtype playing important roles in the response against extracellular pathogens. The differentiation of Th17 cells from naïve T cells is triggered by the concomitant presence of IL6 produced by innate immune cells and TGFβ1 upon TLR stimulation. TGFβ drives the expression of both Rorγt and FoxP3 transcription factors, but IL6-mediated STAT3 activation suppresses FoxP3 expression favouring accumulation of Rorγt (Atarashi et al., 2011b). Rorγt is the master transcription factor in Th17 cells and drives the production of IL17A and IL17F. Rorα transcription factor acts concomitantly to Rorγt favouring the expression of Th17 specific cytokines, even though its expression is not fundamental to the process (Atarashi et al., 2011b). IL21 is another cytokine produced by Th17 cells that sustains and amplifies IL17 expression and leads to Th17 expansion. Moreover IL21 upregulates IL23 receptor (IL23R). IL23 is important for Th17 survival and maturation to a full inflammatory phenotype. IL6, IL21 and IL23 all signal through Stat3 (Atarashi et al., 2011b; Zhu and Paul, 2008). Th17 cells do also

produce IL22 leading to the production of AMPs and epithelial cell proliferation. Stimulation through aryl hydrocarbon receptor (AhR) boosts IL22 production by Th17 cells (Atarashi et al., 2011b).

Th17 cells constitute a minor percentage of the total CD4+ population in the mLNs and PPs, while they are more abundant in the intestine where they represent the 20% and 10% of the CD4 expressing cells in the SI and colon respectively. These numbers suggest that Th17 cells probably differentiate directly in the lamina propria and they are important in intestinal homeostasis. Mice reared in germ free (GF) conditions or subject to antibiotic treatment have reduced Th17 cell percentages, pointing to a role of the microbial community in Th17 differentiation (Atarashi et al., 2011b). The role of the commensal microbiota in the development of Th17 compartment will be discussed in paragraph 1.2.3.1.

1.1.8 Treg cells

Tregs cell have the fundamental role of shutting down inflammatory responses and avoiding improper activation of immune responses. In the absence of proinflammtory cytokines TGF-β drives Smad3 activation that concomitantly with TCR-induced NFAT cause the remodelling of the promoter of the *FoxP3* gene resulting in its expression. Interestingly FoxP3 inhibits Rorγt-dependent IL17 transcription through direct binding and thus blocks Th17 commitment (Atarashi et al., 2011b). Tregs express CD25, glucocorticoid induced TNF receptor family related gene (GITR), and cytotoxic T-lymphocyte antigen 4 (CTLA4) (Zhu and Paul, 2008). Treg cells express IL10, fundamental in mediating their regulatory effects. IL10 is expressed also by another subset of FoxP3 negative cells called Tr1 (Atarashi et al., 2011b).

On the basis of their ontogenesis regulatory T cells are distinguished in natural regulatory T cells (nTregs) that develop in the thymus and are involved in the regulation of auto-reactive T cells in the periphery and inducible regulatory T cells (iTregs) that develop at peripheral sites upon exposure to IL2 and TGF-β (Lin et al., 2013). The most common markers used to distinguish these two Treg subsets are Helios, a member of

Ikaros transcription factor family, and Neuropilin 1, a receptor for vascular endothelial growth factor (VEGF) family members, both of which are prevalently expressed in the nTreg compartment (Lin et al., 2013).

TSLP produced by epithelial cells or DCs is important in tuning the Tregs-Th17 balance thus giving fundamental contribution to intestinal homeostasis. In fact LPS activated DCs produce high levels of TSLP that limit Th17 and promotes Tregs differentiation resulting in containment of inflammation and protection from colitis (Spadoni et al., 2012).

The role of the commensal microbiota and microbial-derived metabolites in the induction of Tregs will be discussed in paragraph 1.2.3.2.

1.2 The intestinal microbiota

Many sites of the human body are inhabited by a multitude of microorganisms. These include the respiratory tract, the skin, the urogenital tract and the intestine. All of these microorganisms are collectively defined to as the microbiota.

The intestinal microbiota has in recent years taken the stage and proved to be of fundamental importance to a number of physiological processes. It is composed by 10^{14} bacteria, exceeding by two orders of magnitude the number of eukaryotic cells in our body, mainly belonging to the phyla Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria (Eckburg et al., 2005; Qin et al., 2010). Even though the kind of relationship that is established between the host and the intestinal microbiota is generally defined commensal it is rather a symbiontic one. In fact the host provides the microbiota with a niche and continuous support of nutrients, while the intestinal microbiota is involved and contributes to many aspects of our homeostasis such as digestive processes, xenobiotic degradation, vitamin metabolism, immune system maturation, defense against pathogens and many others (Eberl, 2010). In this view the host and the microbiota can be seen as a single functional unit, a “superorganisms” that

assembles the genetic endowment and the metabolic capacity of both and has better fitness that the two separate components alone (Eberl, 2010).

It is well proven that the immune system and the microbiota are intimately connected in the intestine and dynamically interact in the maintenance of the homeostatic equilibrium. In fact the immune system maintains the containment and balance of the microbiota with the production of mucus, AMPs, SIgAs and cytokines but can promptly develop an inflammatory response once the equilibrium is broken to re-establish homeostasis. Moreover within this frame microorganisms cannot be defined as good or evil. Pathogenicity results as a combination of host and microbial factors and every microorganism can become a pathogen depending on the context. This concept has given rise to the definition of pathobiont (Eberl, 2010).

1.2.1 The composition of the intestinal microbiota

The composition of the intestinal microbiota is influenced by a number of factors among which diet, antibiotic treatment, genetics of the host and infection are only the most relevant.

The infant gut is sterile at birth, but is promptly colonized by bacteria upon delivery and in the initial phases of life. The delivery route has an important role in defining the "pioneer" microbiota, with vaginally delivered infants having a microbiota most similar to the mother's vaginal microbiota and cesarean delivered infants having intestinal communities more similar to the mother's skin microbiota (Dominguez-Bello et al., 2010; Kelly et al., 2007). After lactation there is a drastic change in microbiota composition, with the anaerobic component becoming prevalent (Kelly et al., 2007; Mackie et al., 1999). Both human and murine intestinal microbiota is dominated by four phyla: Bacteroidetes, Firmicutes, Proteobacteria and Actinobacteria. Together Bacteroidetes and Firmicutes constitute more that 90% of the intestinal bacterial community (Eckburg et al., 2005; Kelly et al., 2007) and unbalances in the Bacteroidetes versus Firmicutes ratio is common to many disorders, as discussed in paragraph 1.2.4. The composition of the intestinal microbiota is conserved between human and mouse at the phylum level,

but there is considerable variation at the species level, with operational taxonomy units (OTUs) in the Firmicutes accounting most of these differences (Chung et al., 2012; Honda and Littman, 2012).

Despite the broad conservation in the composition of the intestinal microbiota at phylum level the MetaHIT consortium has shown that there is high interindividual variation, with only 35% of bacterial genes shared on average among individuals (Eckburg et al., 2005; Honda and Littman, 2012; Qin et al., 2010). The interindividual variation in the microbiota composition is however not a continuum and study from the MetaHIT consortium has characterized three enterotypes by clustering and principal component analysis (Arumugam et al., 2011). Interestingly the three enterotypes differ also in their metabolic potential implying the use of different energy sources among those available in the intestinal lumen (Arumugam et al., 2011).

Diversity is a fundamental characteristic of the intestinal microbiota as pinpointed by evidences that IBD is associated with a reduced microbial diversity, as discussed in paragraph 1.2.4 (Narushima et al., 2014; Qin et al., 2010). Moreover, Chung and colleagues have demonstrated that coevolution of the host and its microbiota has selected a host-specific community that has maximal efficacy in promoting the maturation of the host immunity and protection from infection. In fact administration of murine microbiota to GF mice is effective in increasing intra-epithelial lymphocytes (IELs) abundance, IgA and AMP secretion and protection against infection with *Salmonella* typhimurium while human or rat microbiota fail in doing so (Chung et al., 2012). Moreover the complexity and diversity of the microbiota have fundamental importance since administration of a single species, namely SFB, is not efficient in promoting full maturation of the immune response at the mucosal level (Chung et al., 2012).

Variations in diet, both long- or short-term, lead to alteration both in the composition of the intestinal microbiota and in its gene expression profile (Turnbaugh et al., 2009). This plasticity aims at better extracting energy and nutrients from diet pointing at the maximal fitness for the superorganism (De Filippo et al., 2010; Honda and Littman, 2012; Turnbaugh et al., 2009). Both human and mice under western diet (high fat and high

carbohydrate content) have higher Firmicutes and lower Bacteroidetes (De Filippo et al., 2010; Turnbaugh et al., 2009). On the other hand, the microbiota from African individuals has higher Bacteroidetes and Actinobacteria and shows higher richness and diversity compared to the European one (De Filippo et al., 2010; Honda and Littman, 2012).

The genetic background can also influence microbial composition. TLR5 deficient mice display metabolic syndrome with hyperphagia and increased body weight. This phenotype is transmissible upon transfer of $Tlr5^{-/-}$ microbiota into wild type mice (Arthur and Jobin, 2013; Honda and Littman, 2012; Vijay-Kumar et al., 2010). Interestingly, deficiency in the regulatory cytokine IL10 leads to the development of spontaneous colitis due to a colitogenic microbiota (Arthur and Jobin, 2013; Arthur et al., 2012). Deficiency in AID leads to altered microbial composition in the SI (Suzuki et al., 2004) as discussed in paragraph 1.1.4 and ob/ob mice, a murine model for obesity, have a dysbiotic microbiota that can transfer the phenotype to WT mice (Turnbaugh et al., 2006) as discussed in paragraph 1.2.4.

The use of antibiotics to target infectious agents has been one of the most striking progresses of modern medicine. However antibiotics are never specific for a given pathogen and rather affect multiple bacterial groups of the endogenous microbiota. Given that the microbiota is a community that relies on multiple interactions the whole community is actually affected. Antibiotic treatment causes a reduction in microbial diversity that is only partially restored upon treatment ceasing (Jernberg et al., 2007; Willing et al., 2011). By affecting microbial homeostasis, antibiotics do also affect host health being the host-microbiota mutualism so fundamental for the superorganism (Willing et al., 2011). Interestingly treatment with the antibiotic metronidazole, which targets the anaerobic component of the gut microbiota, affects MUC2 expression and mucus layer integrity, allowing bacteria to penetrate deeper in intestinal crypts (Pelissier et al., 2010; Willing et al., 2011).

The intestinal microbiota has a complex and dynamic composition, but this is largely exceeded by the complexity of the metabolic capacity of the microbiota. The metabolites

produced by the microbiota, collectively referred to as microbial metabolome, have fundamental functions in the host homeostasis and immune system maturation (Dorrestein et al., 2014). The complexity of the microbial metabolome and its influences on the host has just started to be elucidated and some keypoints regarding microbial influence on host immune homeostasis will be described in the following paragraphs.

1.2.2 Influence of the microbiota on the mucus barrier

The intestinal microbiota plays a fundamental role in the development and maturation of the intestinal barrier and immune system. Gnotobiotes, animal models with a defined microbiological status, have been fundamental tools to understand how specific microbes can sustain the development of specific immune cell populations.

In GF animals the mucus layer is thinner, less compact and differentially glycosylated when compared to conventionally reared mice (Petersson et al., 2011). This suggests that intestinal microbes play a role in the maintenance of an intact mucus barrier. Consistently treatment with bacterial products LPS and PGN restores normal mucus thickness (Petersson et al., 2011). Further experimental evidence shows that the expression of mucin genes is upregulated in GF mice compared to conventional ones and fecal transplantation in GF mice restores normal mucin expression levels (Comelli et al., 2008). Interestingly fecal transplantation of human microbiota is unable to downmodulate mucin genes as the murine one does, underlining another host-specific aspect related to the microbiota as discussed in paragraph 1.2.1 (Comelli et al., 2008). Moreover, as discussed also in paragraph 1.1.1.1, an increase in mucus secretion is a mechanism of pathogen clearance as demonstrated by the increased MUC2 secretion upon *Citrobacter rodentium* infection (Petersson et al., 2011).

1.2.3 Influence of the microbiota and its metabolites in intestinal immune system development

GF animals have smaller PPs and mLNs compared to conventionally reared mice. Moreover they have lower numbers of IELs, plasmacells and reduced production of IgAs and AMPs. Neonates at birth have PPs and mLNs, but the massive bacterial colonization happening at birth and in the first phases of life induces enlargement and maturation of these organs. ILFs, on the contrary, do only develop in the presence of the intestinal microbiota (Atarashi et al., 2011b; Eberl, 2010). These were the first evidences that microbial presence and compositions were fundamental in shaping the gut resident immune populations. In the following paragraphs the importance of some microbial groups in shaping the two most abundant populations in the intestinal lamina propria, namely Th17 and Tregs, will be discussed.

1.2.3.1 SFB and Th17 development

Ivanov and colleagues demonstrated that a particular component of the murine commensal microbiota controls the abundance of Th17 and Tregs in the SI lamina propria through a TLR-independent mechanism. In fact mice reared in GF conditions have very few Th17 cells but almost doubled Treg population compared to conventional mice. Moreover treatment of specific pathogen free (SPF) mice with ampicillin or vancomycin reduces Th17 abundance in the intestine. Cohousing of GF or antibiotic treated mice with SPF mice restores normal Th17 and Tregs proportions, pointing to a role for a subset of bacteria rather than the whole microbial community in determining the differentiation of Th17 cells in the SI lamina propria (Ivanov et al., 2008). Subsequently 16S metagenomic analysis of the ileal content of Jackson B6 mice (low Th17 abundance in the SI) versus Taconic B6 mice (high Th17 abundance in the SI) highlighted the correlation between SFB with Th17 abundance. SFB strictly adhere to the surface of IECs (Gaboriau-Routhiau et al., 2009; Ivanov et al., 2009). Transcriptional profiling highlighted the induction of immune and inflammatory transcriptional

programmes in the ilei of germ free mice reconstituted with SFB (Gaboriau-Routhiau et al., 2009; Ivanov et al., 2009). The most upregulated gene encodes for serum amyloid A (SAA) protein that could be secreted by IECs upon SFB stimulation and condition lamina propria DCs to favour Th17 differentiation. SFB colonized mice are also more resistant towards *Citrobacter rodentium* infection, pointing at the fundamental role of Th17 cells in the intestinal immune homeostasis (Ivanov et al., 2009). Mechanistically intestinal DCs acquire antigens from SFB strictly adherent to IECs. DCs then present SFB antigens in major histocompatibility complex class II (MHCII) context to induce SFB specific Th17 cells (Goto et al., 2014b).

1.2.3.2 Clostridia and Tregs induction

Tregs are the other T cell subset present in the intestinal lamina propria. Treg cell percentages are not altered in the small intestine of GF mice, while they are strongly reduced in the colon. Notably, in mice the abundance of Treg cells in the colon dramatically increases after weaning pointing to a possible correlation with microbial stimulation. Indeed some *Clostridium* species are strong inducers of IL10 expressing Treg in the colonic lamina propria (Atarashi et al., 2013; Atarashi et al., 2011a). Atarashi and colleagues identified and characterized a mixture of 17 strains in the chloroform-resistant (spore-forming) fraction of the human microbiota by means of 16S rDNA sequencing. These strains belonging to Clostridial classes VI, XIVa and XVII and are sufficient for colonic Tregs induction in GF mice. The different strains somehow synergize since the 17 strains taken individually fail in inducing Tregs and a core mix of 5 strains leads to significant increase of colonic Tregs even if still lower than the one elicited by the 17 strains mix. The 17 strain treatment shows efficacy *in vivo* against different models of murine colitis. The Clostridia strains produce short chain fatty acids (SCFA), namely acetate, propionate, butyrate and isobutyrate that induce TGFβ1 in colonic epithelial cell lines (Atarashi et al., 2013). Among SCFAs, butyrate is the one able to induce Tregs *in vitro*, *in vivo* and in experimental colitis, and the 17 strains have a number of genes involved in butyrate metabolism (Narushima et al., 2014). Butyrate

acts at the epigenetic level increasing the acetylation level, and thus accessibility to the transcriptional machinery, of the *FoxP3* locus and related enhancer elements suggesting a possible mechanism for Tregs induction (Furusawa et al., 2013). GF mice have reduced levels of SCFAs, highlighting the importance of the microbiota in their biosynthesis (Brestoff and Artis, 2013).

Apart from inducing iTreg cell differentiation SCFAs have effects also in other immune cell types. In fact SCFAs stimulate neutrophil chemotaxis and activation, and elicit anti-inflammatory response in DCs as well as in the epithelial cell line Caco-2. All these effects are mediated by the G-protein coupled receptor GPR43 (Brestoff and Artis, 2013).

The importance of intestinal microbes in the induction of Tregs and maintenance of homeostasis has also been addressed by Geuking and colleagues who observed that altered Schaedler flora (ASF), a mixture of eight benign commensals, results in the induction of colonic Treg cells that are fundamental in regulating Th1 and Th17 responses (Geuking et al., 2011). Single components of the ASF failed in inducing Tregs as did the single Clostridial species studied by Atarashi and colleagues (Atarashi et al., 2013) highlighting that also in this case synergy and interaction among bacterial species are fundamental for host physiology (Geuking et al., 2011).

1.2.3.3 The microbiota and bile acid metabolism

The intestinal microbiota takes part in bile acid metabolism. Bile acids are synthetized in the liver, stored in the gall bladder and released in the duodenum upon transit of the bolus. Once in the gut primary bile acids are converted into secondary bile acids through dehydration reactions that are peculiar to the microbiota. Secondary bile acids are then reabsorbed in the ileum through the enterohepatic circulation. In the liver they are conjugated to glycine and taurine to be secreted again in the intestine. Bacteria do also take part in another important step of bile acid metabolism that is the deconjugation of secondary bile acids that leads to secretion, thus regulating their turnover (Brestoff and Artis, 2013). Studies with GF or antibiotic-treated animals demonstrated that in the

absence of microbiota there are profound changes in bile acid profiles in the liver, intestine and faeces and also at distal sites such as the kidney, heart and plasma (Sayin et al., 2013; Swann et al., 2011). Bile acids can start signalling cascades through binding to the membrane G protein-coupled bile acid receptor 1 (GPBAR1) and the nuclear farnesoid X receptor (FXR). The microbiota does also participate in the regulation of bile acid synthesis in the liver through an FXR dependent mechanism (Sayin et al., 2013). Moreover in a number of immune cells including monocytes, macrophages and DCs bile acid signalling is associated with an anti-inflammatory response mediated by the repression of NF-kB dependant transcriptional response. The anti-inflammatory effect of bile acid can probably be translated also to the non-immune compartment, as bile acid signalling elicits anti-inflammatory response also in the epithelial cell line Caco2 (Brestoff and Artis, 2013).

1.2.3.4 The microbiota and xenobiotic metabolism

The intestinal microbiota has a fundamental role also in xenobiotic metabolism (Eberl, 2010). Recent experimental data demonstrated that the intestinal microbiota is required for chemotherapeutic efficacy towards solid tumors (Iida et al., 2013; Viaud et al., 2013). Notably the alkylating agent cyclophosphamide elicits dysbiosis in the small intestine and provokes the translocation of a population of gram-positive bacteria to secondary lymphoid organs, where they induce a pathogenic Th17 population that mediates tumor regression. The development of the pathogenic Th7 population is dependent on MyD88, directly linking their development to bacterial sensing. In the absence of the intestinal microbiota pathogenic Th17 cells do not expand and cyclophosphamide is not efficient in mediating tumor regression (Viaud et al., 2013). Further evidence involves the chemotherapeutic oxaliplatin, whose mechanisms of action involve reactive oxygen species (ROS) production by myeloid cells. Oxaliplatin mediated tumor regression is dependent on MyD88 and antibiotic treatment dampens the efficacy of the treatment (Iida et al., 2013).

1.2.4 The microbiota in disease

Aberrant immune responses towards the microbiota or improper sensing of its components are associated with pathological state. Importantly, chronic inflammation often leads to reduced diversity compared to healthy individuals, with IBD patients having a 25% microbial gene reduction (Qin et al., 2010). Maintenance of microbial diversity is fundamental to perpetuate intestinal and systemic homeostasis (Honda and Littman, 2012).

IBD patients have an increase in adherent-invasive *Escherichia coli* that physically adhere and invade the ileal IECs (Darfeuille-Michaud et al., 2004). Moreover these patients have a reduction in both Bacteroidetes and Firmicutes while Proteobacteria and Actibacteria do increase (Atarashi et al., 2011b; Frank et al., 2007; Honda and Littman, 2012). IBD patients do also have reduced proportion of butyrate-producing bacteria and a reduced expression of butyrate transporter in colonic epithelial cells. Exogenous administration of butyrate ameliorates inflammation in IBD patients suggesting that butyrate depletion might play a role in IBD pathogenesis (Brestoff and Artis, 2013; Dorrestein et al., 2014; Furusawa et al., 2013; Thibault et al., 2010; Thibault et al., 2007). IBD-associated microbiota is also characterized by reduced diversity when compared to healthy patients (Qin et al., 2010). The 17 Treg-inducers Clostridial strains isolated from human healthy microbiota (Atarashi et al., 2013) have considerable heterogeneity in terms of metabolic properties and this together with the observation that they can ameliorate experimental colitis points to the fundamental role of microbial diversity in intestinal homeostasis and to the important therapeutic value of restoring diversity upon dysbiosis (Narushima et al., 2014).

The *CARD15* locus, encoding the NOD2 receptor, is frequently mutated in Crohn's disease patients (Lesage et al., 2002). These mutations can either increase NOD2 activity with consequent hyperactivation of Nf-κB and IL1β secretion or abolish NOD2 activation with consequent decrease of AMPs secretion. This last scenario leads to increased bacterial stimulation through TLR2 (Strober et al., 2008). As a result both kinds of mutation result in hyperresponsiveness towards the microbiota (Eberl, 2010).

Another disease in which the presence of dysbiosis is well documented is obesity (Turnbaugh et al., 2006). Even if the mechanisms lading to the establishment of such a dysbiosis are not clear obese patients have an increased proportion of Firmicutes versus Bacteroidetes and a general reduction in bacterial richness (Irrazabal et al., 2014; Turnbaugh et al., 2006). The obese microbiota is more efficient than normal in extracting energy from nutrients and in particular from polysaccharides and studies in murine models demonstrate that the obese phenotype is transferrable upon fecal transplantation from ob/ob mice (deficient in the leptin gene) to wild type GF animals (Turnbaugh et al., 2006). This raises an important point regarding the inheritance of the disease (Eberl, 2010).

The contribution of the microbiota in the severity of type-1 diabetes was elucidated with NOD murine models deficient in MyD88 (Wen et al., 2008). MyD88$^{-/-}$NOD mice are resistant to diabetes development and have lower diabetogenic T cells in the pancreatic lymph nodes. Moreover the MyD88$^{-/-}$ microbiota (that has a low Firmicutes versus Bacteroidetes ratio) is protective towards diabetes development in NOD mice (Eberl, 2010; Wen et al., 2008).

Current knowledge about dysbiosis in colorectal cancer will be discussed in paragraph 1.3.4.

1.3 Colorectal cancer

Colorecral cancer is a leading cause of cancer related death and is therefore a major health problem. From the aetiological point of view CRC is a multifactorial disease, with genetic component, diet contribution and inflammation interacting and resulting in the disease. In the last years also intestinal microbiota has been claimed to participate in CRC aetiology.

1.3.1 The genetics of colorectal cancer

Colorectal cancer originates from intestinal adenomas that progress to invasive carcinoma and ultimately metastasize distal sites as shown in Figure 1-4 (Irrazabal et al., 2014; Markowitz and Bertagnolli, 2009).

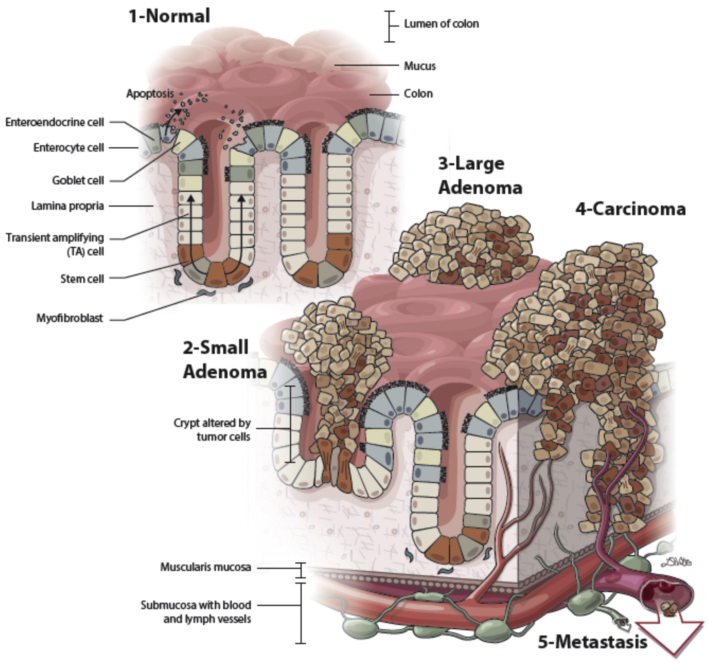

Figure 1-4 Colorectal cancer develops from benign adenomas that progress to invasive carcinomas and ultimately metastatize to distal organs. Adapted from (Irrazabal et al., 2014).

Hereditary CRC accounts only for 10% of the cases. Familial adenomatous polyposis (FAP) is an autosomal dominant syndrome characterized by germline mutations in the adenomatous polyposis (apc) gene. FAP patients develop massive polyposis in the GI tract during adolescence and have 100% risk of developing CRC by the age of 40 (Bogaert and Prenen, 2014). Hereditary non-polyposis colorectal cancer (HNPCC), also known as Lynch syndrome, is the most frequent hereditary CRC form accounting for 2-5% of cases. It is characterized by germline mutations in mismatch repair genes that lead to microsatellite instability (Bogaert and Prenen, 2014).

In 1990 Fearon and Vogelstein described the genetic alterations leading to sporadic CRC as a multistep process that confers a growth advantage to transformed colonocytes

(Fearon and Vogelstein, 1990). More recently genome-wide sequencing data identified an average of 80 mutated genes per tumor, even if less that 15 of these are considered driver mutations in the pathogenesis (Bogaert and Prenen, 2014; Markowitz and Bertagnolli, 2009).

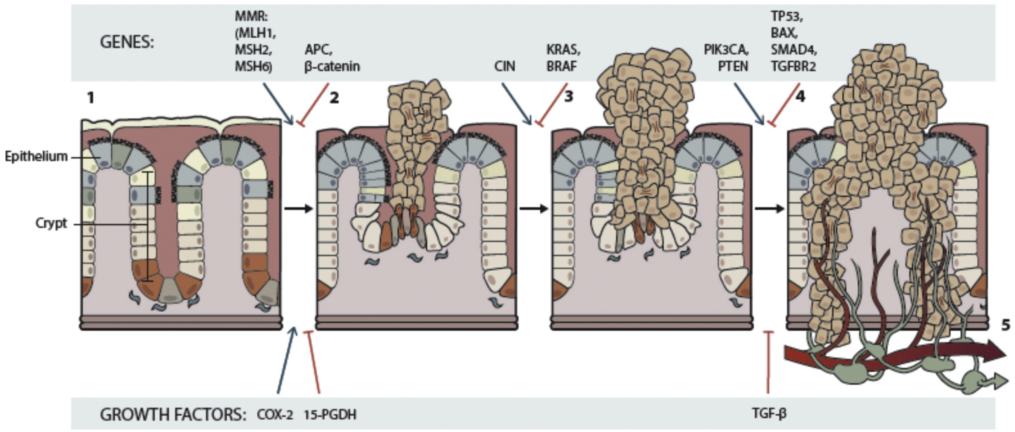

Figure 1-5 The genetics of CRC. The most common initiator events are mutations in APC and in mismatch repair genes. Subsequent accumulation of mutations leads to progression and development of cancer. Adapted from (Irrazabal et al., 2014).

There are three main pathways leading to sporadic CRC: chromosomal instability, microsatellite instability and CpG island methylation pathway.

Chromosomal instability is the most common initiator event accounting for 70% of cases. It is characterized by chromosomal rearrangements and loss of heterozigosity (LOH). Among the oncogens and oncosuppresor genes that are frequently mutated we find APC, KRAS, SMAD4, TP53, BRAF and PIK3CA (Bogaert and Prenen, 2014; Markowitz and Bertagnolli, 2009) and it is the concomitant presence of these mutations rather than their temporal sequence that determines transformation (Fearon and Vogelstein, 1990).

Microsatellite instability is caused by mutation in mismatch repair genes that results in aberrant microsatellite DNA insertion or deletions and subsequent frameshift mutations in the coding regions (Bogaert and Prenen, 2014; Markowitz and Bertagnolli, 2009).

CpG island methylation pathway results in aberrant DNA methylation status of CpG dinucleotides in the promoter regions of some genes that leads to the epigenetic

silencing of such genes. This is paralleled by a general hypomethylation status of the colorectal genome (Markowitz and Bertagnolli, 2009).

Moreover, microRNA (miRNA)-mediated modulation of gene expression has been investigated as a further epigenetic mechanism contributing to CRC. Consistently some miRNAs such as miR-21, miR-106 and miR-144 are more abundant in faecal samples from CRC patients compared to controls and can be used as biomarkers for CRC (Yang et al., 2013).

Activation of the Wnt signalling pathway (Figure 1-6) is considered the initiator event in CRC pathogenesis (Markowitz and Bertagnolli, 2009). Wnt signalling culminates in the nuclear translocation of the transcription factor β-catenin and the expression of genes related to cellular proliferation. In differentiated cells the cellular level of β-catenin is controlled by the β-catenin degradation complex that targets it for proteolytic degradation. APC is part of the β-catenin degradation complex and its inactivation leads to increased β-catenin levels and expression of β-catenin related genes.

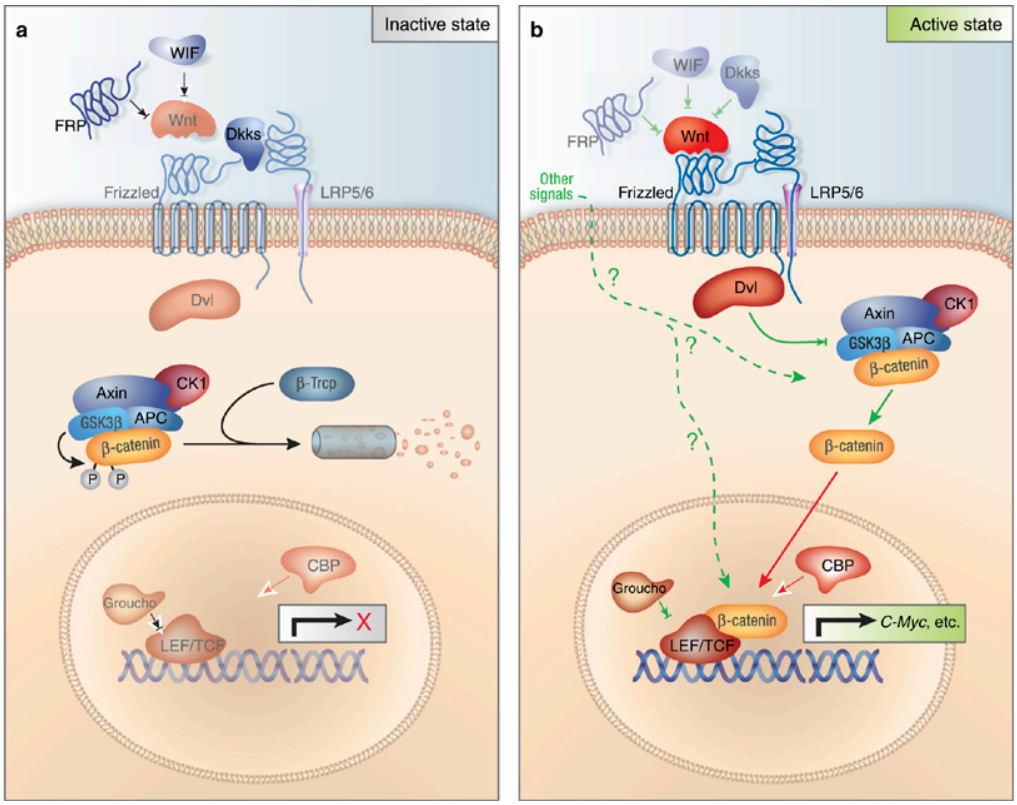

Figure 1-6 Wnt signalling pathway. a) In the absence of wnt signal β-catenin is phosphorylated by the β-catenin degradation complex and targeted for proteasome-mediated cleavage in the cytosol. In this way β-

catenin is excluded from the nucleus. b) When wnt ligand binds to frizzled receptor the β-catenin degradation complex disaggregates. This allows β-catenin migration in the nucleus and activation of a transcriptional response. Adapted from (Luo et al., 2007).

Mutations in APC are a characteristic of both hereditary and sporadic CRC, being found in nearly 80% of cases. Less common are mutations that render β-catenin resistant to degradation and thus constitutively active (Anastas and Moon, 2013; Markowitz and Bertagnolli, 2009).

1.3.2 Mucus alterations in colorectal cancer

As described in paragraph 1.1.1.3 for IBD, mucin expression and glycosylation is altered in CRC.

MUC1 is described as an oncogene in breast cancer where it is frequently overexpressed and in many cases also amplified at the genomic level (Lacunza et al., 2010) and its expression is frequently enhanced in hCRC (Ajioka et al., 1996). The intracellular domain of MUC1 interacts with β-catenin and its phosphorylation disrupts these interactions linking MUC1 with the Wnt signalling pathway, the key player in CRC (Sheng et al., 2012). Moreover by binding β-catenin MUC1 is also involved in the dismantling of adherens junctions, and thus possibly in epithelial to mesenchymal transition (Sheng et al., 2012). In addition to this the overexpression of large membrane-bound mucins by cancer cells, as is MUC1 in breast cancer, is associated with topological rearrangements in the plasma membrane involving integrins and adhesion molecules. This is a merely physical process not involving MUC1 signalling properties but confers to cancer cells a survival advantage pointing to a contribution of this process in epithelial to mesenchymal transition and invasion (Paszek et al., 2014).

The importance of a proper MUC2 expression is underlined by the observation that $Muc2^{-/-}$ mice spontaneously develop CRC (Velcich et al., 2002), as already discussed in paragraph 1.1.1.1. Moreover APC-initiated CRC often displays goblet cell depletion. Lack of Muc2 exacerbates tumorigenesis in the $Apc^{Min/+}$ mice in a dose-dependant

manner in all the intestinal tracts, but with a particular penetrance in the colon (Yang et al., 2008). Lack of Muc2 unmasks microbial inflammatory stimuli, as documented by an increased expression of AMPs such as RegIII-γ (Yang et al., 2008). The phenotype observed in $Muc2^{-/-}Apc^{1638N/+}$ mice is similar to the one observed by Tanaka and colleagues, who were the first showing that DSS acts as a promoting agent in the $Apc^{Min/+}$ context (Tanaka et al., 2006). DSS treatment significantly increases the number of small intestinal adenomas and profoundly affects tumorigenesis in the colon (Tanaka et al., 2006).

1.3.3 Inflammation/immune cells in colorectal cancer

Inflammation has since a long time been associated with tumorigenesis and CRC is one of the cancer types where the involvement of inflammation is widely proven.

The proinflammatory cytokine IL17A has a tumor promoting role in the $Apc^{Min/+}$ model and its genetic ablation reduces the tumor load in both the SI and colon (Chae et al., 2010). Additional work in animal models of APC-driven tumorigenesis has shown that loss of barrier function at the tumor site leads to microbial stimulation and entry causing recruitment of IL23-producig myeloid cells. IL23 stimulates recruitment and differentiation of Th17 and other IL17A producing cells that create a proinflammatory environment that favours tumor growth (Grivennikov et al., 2012). Recent work has proven the role of another member of the IL17 cytokine family in intestinal tumorigenesis. IL17C signalling through IL17RE on IECs promotes cell survival and proliferation and hence tumorigenesis in a MyD88 dependant fashion (Song et al., 2014).

Murine models have shown that absence of the suppressive cytokine IL10 leads to chronic colitis as a result of exacerbated T cell response. This inflammatory status acts as tumor promoter when *Il10*$^{-/-}$ mice are challenged with the initiator AOM (Arthur and Jobin, 2013). *Il10*$^{-/-}$ mice have altered intestinal microbial community if compared to WT mice but interestingly AOM/*Il10*$^{-/-}$ mice do not have dysbiosis if compared with *Il10*$^{-/-}$ counterparts, meaning that the promoting agent in this model is inflammation rather than dysbiosis (Arthur and Jobin, 2013). However, inflammation *per se* is not sufficient to

trigger tumorigenesis as demonstrated by the fact that *E. coli* NC101 and *Enterococcus faecalis* both induce severe colitis in monoassociated *Il10*$^{-/-}$ mice with similar infiltrates and cytokine profiles, but only the first can induce tumorigenesis in the AOM/*Il10*$^{-/-}$ model (Arthur and Jobin, 2013; Arthur et al., 2012).

The role of Treg cells in intestinal tumorigenesis is controversial. Studies in the Apc$^{\Delta 468/+}$ mice show that there is a massive recruitment of Tregs in the polyps and that these cells are highly suppressive *in vitro*, but they are defective in the production of the cytokine IL10 and rather express a Th17-like signature (Gounaris et al., 2009). These proinflammatory Tregs promote polyposis, while adoptive transfer of nTregs from WT mice represses it (Gounaris et al., 2009). Treg cells isolated from polyps show contemporary expression of FoxP3 and Rorγt transcription factors and are enriched in UC and colitis associated cancer (Blatner et al., 2012). Rorγt rather than IL17 expression confers these cells their pathogenic role in polyposis (Blatner et al., 2012). Recent work highlighted that sustained β-catenin signalling in CD4+ and FoxP3+ cells is driving Rorγt expression conferring them the proinflammatory phenotype and that CD4+ T cells with constitutively active β-catenin signalling are able to induce intestinal inflammation and tumorigenesis (Keerthivasan et al., 2014).

1.3.4 The microbiota in colorectal cancer

CRC implies as initiator events the genetic alterations described in paragraph 1.3.1, but environmental factors can play a role in tumor progression (Zhu et al., 2013). The GI tract is pervasively colonized by microorganisms and the interactions occurring between the tumor and the microbiota upon tumorigenesis have been investigated at different levels in the last years. Even if there is high heterogeneity in the nature of samples analysed and techniques used two points emerge clearly from the present literature: first there is a dysbiosis associated with CRC and second bacterial metabolites can be involved in tumorigenesis (Zhu et al., 2013).

Primary evidence that the intestinal microbial community plays a role in intestinal tumor progression comes from animal models that display reduced disease penetrance if

reared in GF conditions or treated with anibiotics (Sears and Garrett, 2014; Zhu et al., 2013). Moreover, deficiencies in PRRs have been shown to attenuate CRC penetrance, implying a role for microbial sensing in disease progression (Zhu et al., 2013).

Sequencing analysis revealed that the microbial communities of cancerous vs. non-cancerous tissue from the same patient cluster together, meaning that the intestinal microenvironment might be more important than the tumor itself in shaping the microbial community (Kostic et al., 2012). However the tumor tissue shows depletion in Firmicutes, particularly in the order Clostridiales, and Bacteroidetes. The genus *Fusobacterium* is enriched in many, even though not all, adenomas, carcinomas and metastases relative to the matched control tissue and also in the feaces of CRC samples (Kostic et al., 2013; Kostic et al., 2012). Moreover, *Fusobacterium nucleatum* exacerbates both small intestinal and colonic tumorigenesis in the $Apc^{Min/+}$ model of genetically driven tumorigenesis without induction of inflammation (Kostic et al., 2013). Adenomas from *F. nucleatum*-trated $Apc^{Min/+}$ mice have higher myeloid derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs) with M2 characteristics, tumor-associated neutrophils (TANs) and DCs infiltrates (Kostic et al., 2013). This is mirrored by a pro-inflammatory Nf-κβ dominated signature that is common with *F. nucleatum*-high human CRC tissues, that is possibly one of the driving forces of tumorigenesis (Kostic et al., 2013). *In vitro* studies suggest that mechanistically *F. nucleatum* binds to E-cadherin through the activated complex of FadA adhesion and activates Wnt- βcatenin signalling cascade (Sears and Garrett, 2014).

The mechanism by which species that are enriched in tumor tissues could be involved in tumorigenesis may imply induction of inflammation, as highlighted by the evidence that $Apc^{Min/+}$ mice chronically colonized with enterotoxigenic *Bacteroides fragilis* have increased STAT3 activation and Th17-driven colonic inflammation and colonic polyposis (Sears and Garrett, 2014; Zhu et al., 2013).

The role of the microbial metabolic activity has been investigated as well and linked to intestinal tumorigenesis. Some enzymatic activities that are peculiar to the microbiota can lead to the transformation or release of genotoxic substances possibly implied in

tumorigenesis. An example of this is β-glucuronidase activity. Toxic compounds are metabolized in the liver and excreted in the form of highly soluble glucuronic acid conjugates. Some bacterial species express β-glucuronidase that hydrolyses glucuronic acid conjugates releasing the toxic compounds in the intestinal lumen. As a matter of facts CRC patients have high β-glucuronidase activity in the faeces (Sears and Garrett, 2014; Zhu et al., 2013).

Intestinal bacteria do play an important role in the metabolism of bile acids, as discussed in paragraph 1.2.3.3. Faecal content of lithocholic and deoxycholic acid are elevated in the faeces of CRC patients and these secondary bile acids potentiate tumorigenesis in murine models of CRC (Sears and Garrett, 2014). On the contrary ursodeoxycholic acid is beneficial in CRC. This bile acid is derived from chenodexycholic acid via two enzymatic activities, 7α-hydroxysteroid dehydrogenase and 7β-hydroxysteroid dehydrogenase, that are peculiar to some bacterial groups, including Clostridia (Sears and Garrett, 2014). Loss of bile acid-induced signalling is detrimental for tumorigenesis as AOM/DSS treated $FXR^{-/-}$ mice and $FXR^{-/-}Apc^{Min/+}$ mice develop more tumors (Modica et al., 2008). One possible player in the FXR mediated attenuation of tumorigenesis is taurocholic acid that does attenuate tumorigenesis in $Apc^{Min/+}$ mice through reducing proliferation and increasing apoptosis in the ileum (Smith et al., 2010).

Additionally, recent work in the $Apc^{Min/+}MSH2^{-/-}$ murine model shows that treatment with a diet with low carbohydrate content reduces intestinal polyposis to a level similar to the one observed when the same mice are kept under antibiotic regimen. The explanation for this is in the reduction in the luminal abundance of a bacterial metabolite, namely butyrate, that promotes epithelial cell proliferation and thus polyposis (Belcheva et al., 2014).

Finally, bacterial metabolism can lead to the production of ROS that can directly act as genotoxic agents modifying DNA structure or sequence and leading to mutations or genetic instability (Zhu et al., 2013). As an example of this *Enterococcus faecalis* superoxide production increases tumorigenesis in the *Il10*$^{-/-}$ murine model, while a strain deficient in superoxide production does not (Sears and Garrett, 2014). Phylogenetic

group B2 *Escherichia coli*, such as the strain NC101 frequently found as intracellular bacteria in hCRC samples, has direct DNA-damaging activity by means of the genotoxin colibactin, and its deletion decreases CRC phenotype when compared to the wild type strain in the *Il10*$^{-/-}$AOM murine model (Arthur et al., 2012) (Arthur and Jobin, 2013) (Sears and Garrett, 2014).

Experimental evidence that the microbial ecosystem is altered upon intestinal tumorigenesis poses interesting issues regarding possible therapeutic interventions employing bacteria or their metabolic products as treatments. The use of probiotics has been shown to have positive effects on the maintenance of microbial equilibrium, inhibition of pathogen colonization and stimulation of host immunity (Kahouli et al., 2013; Zhu et al., 2013). In fact probiotic administration has been shown effective in strengthening the epithelial barrier by preventing epithelium leakage and improving mucus secretion (Kahouli et al., 2013). Moreover re-balancing the intestinal bacterial ecosystem can both counteract carcinogenic enzymatic activities and remove their toxic products (Kahouli et al., 2013). Probiotics can reduce the cancer-associated genotoxic microenvironment and induce apoptosis while preventing proliferation of colonic ECs (Kahouli et al., 2013). Although limited, studies on the use of probiotics concomitantly to classical chemotherapy report beneficial effects of the treatment on the health status of patients (Kahouli et al., 2013).

2 AIM OF THE WORK

The study of the intestinal microbiota and its influence on host physiology has become a burning field of investigation in recent years. In particular, the influence of the microbiota on tumor development is an intriguing field of research.

In this work using the $Apc^{Min/+}$ murine model we investigated how the microbiota could influence intestinal tumorigenesis and whether dysbiosis could somehow influence the progression of tumors, possibly mediated by alterations in the mucus layer. Finally we addressed whether bacterial stimuli could alter *apc* gene expression contributing to tumor progression.

The specific points we have addressed are the following:

- Verify if the $Apc^{Min/+}$ model of intestinal tumorignesis has alterations in the mucus layer as happens in the human pathology;
- Address whether alterations in the mucus barrier result in altered barrier properties during tumorigenesis;
- Analyse how exogenous alterations of the mucus barrier impact on tumorigenesis;
- Analyse the evolution of the intestinal microbial community during tumorigenesis;
- Address the possible functional impact of species that are differentially abundant between wild type and $Apc^{Min/+}$ mice for the tumorigenic process;
- Investigate whether bacterial stimuli can modulate *apc* gene expression contributing to tumor progression.

3 MATERIALS AND METHODS

3.1 Mice

6 weeks old C57BL/6J female mice were purchased from Harlan Laboratories. C57BL/6J-Apc^{Min}/J (referred to as Apc$^{Min/+}$) mice were obtained as a result of genetic mutation induced by treatment with N-ethyl N-nitrosurea. T to A transversion at position 2549 of the *apc* gene causes the generation of a premature stop codon. These mice develop multiple intestinal neoplasia (min) characterized by adenomas in the small intestine and colon (Moser et al., 1990). Apc$^{Min/+}$ mice colony is maintained as heterozygous in our animal facility by breeding Apc$^{Min/+}$ males with C57Bl6/J females purchased from Harlan. At weaning mice are screened for the presence of the min allele by multiplexed PCR on genomic DNA using primers listed in Table 3-1.

Primer	Sequence (5'→3')	Allele recognized
oIMR0033	GCCATCCCTTCACGTTAG	Wild type
oIMR0034	TTCCACTTTGGCATAAGGC	Common
oIMR0758	TTCTGAGAAAGACAGAAGTTA	Mutated

Table 3-1 Primer sequences for Apc$^{Min/+}$ genotyping assay

The cycling protocol used is described in Table 3-2.

Temperature (°C)	Time	N. of cycles
94	3 minutes	1
94	30 seconds	x 35 cycles
55	1 minute	
72	1 minute	
72	2 minutes	1

Table 3-2 Thermal cycler protocol used for Apc$^{Min/+}$ genotyping assay

The assay amplifies a 600 bp band for the wild type allele and a 340 bp band for the min allele as shown in Figure 3-1.

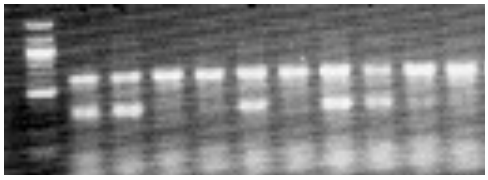

Figure 3-1 $Apc^{Min/+}$ genotyping assay. The gel shows bands for the wild type and min apc allele.

Wild type mice used for experiments are littermates from our $Apc^{Min/+}$ mice colony.

All mice were maintained in microisolator cages in a specific pathogen-free animal facility and experiments were performed in accordance with the guidelines established in the Principles of Laboratory Animal Care (directive 86/609/EEC) and approved by the Italian Ministry of Health.

3.2 FACS on organs

To analyse the principal immune cell population peripheral blood and mLN were sampled from WT and $Apc^{Min/+}$ mice at 8, 12, 16 and 20 or more weeks of age. Red blood cells were lysed and mLNs were reduced to single cell suspensions. Neutrophils were stained with Ly6G PE (clone 1A8, BD Biosciences) and CD11b APC (clone M1/70, BD Biosciences). T cells were stained with CD4 FITC (clone RM4-4, BD Biosciences) and CD8 APC (clone 53-6.7, BD Biosciences). B cells were stained with B220 FITC (clone RA3-6B2, BD Biosciences) and CD19 APC (clone 1D3, BD Biosciences). Samples were acquired at FACSCalibur cytometer (BD Biosciences). Populations are considered as percentage on living cells identified with SSC and FSC.

To detect neutrophils and inflammatory monocytes in circulating blood of mice treated or not with *Clostridium* ID4 blood was sampled in heparin *via* tail vein bleeding. Erythrocytes were lysed with red blood cell lysis buffer (6 mM NH_4Cl, 140 mM $NaHCO_3$, 16 mM EDTA, pH 7,4). Cells were stained with CD45.2 PerCP-Cy5.5 (clone 104, eBioscience), CD3 PE-Cy7 (clone SK7, BD Biosciences), Ly6G PE (clone 1A8, BD

Biosciences), Ly6C FITC (clone AL-21, BD Biosciences) and CD11b APC (clone M1/70, BD Biosciences). Samples were acquired at FACSCantoII (BD Biosciences). Inflammatory monocytes were identified as CD11b+ Ly6C^{high} cells in the CD45+ CD3- gate, while neutrophils were identified as CD11b+ Ly6G+ cells in the CD45+ CD3- gate.

3.3 RT-qPCR assay

Intestinal chunks from wild type mice, normal ileal chunks and single polyps from Apc$^{Min/+}$ mice were sampled at 10 and 23 weeks of age. Intestinal tissue was homogenised in 500 ul of TRIzol (Invitrogen). RNA was extracted adding 100 µl of chloroform, precipitating the aqueous phase with 300 µl of 70% ethanol and purifying RNA with RNeasy Mini Kit (QIAGEN) according to the manufacturer's guidelines. Genomic DNA contamination was removed with DNase-Free DNase set (QIAGEN). RNA was retro-transcribed with ImProm-II Reverse Transcriptase kit (Promega) following manufacturer's instruction. qPCR assay was performed with Fast Sybr Green Master Mix (Life Technologies) on 10 ng cDNA template/reaction using exon-spanning primers (final concentration 1 µM).

To isolate RNA from single ileal crypts formalin-fixed swiss rolls from wild type and Apc$^{Min/+}$ mice aged 16 weeks underwent laser-assisted microdissection, allowing fine collection of both normal and dysplastic crypts in the Apc$^{Min/+}$ ilei. Laser-assisted microdissection was performed in Prof. Silvano Bosari laboratory (Department of Patophisiology and Transplantation, University of Milan, Italy). RNA was retrotranscribed with High Capacity cDNA Reverse Transcription Kit (Life Technologies) and qPCR assay was performed on 10 ng cDNA as previously described.

Expression levels are normalized to the 60S ribosomal protein gene espression RPL32.

Primers used are listed in Table 3-3.

Gene	Forward primer (5'→3')	Reverse primer (5'→3')	Amplicon (bp)
Rpl32	AAGCGAAACTGGCGGAAAC	TAACCGATGTTGGGCATCAG	86
Muc1	TACCCTACCTACCACACTCACG	CTGCTACTGCCATTACCTGC	95
Muc2	CACCAACACGTCAAAAATCG	GGTCTCTCGATCACCACCAT	116
Muc3	CTTCCAGCCTTCCCTAAACC	TCCACAGATCCATGCAAAAC	119
Muc4	GAGAGTTCCCTGGCTGTGTC	GGACATGGGTGTCTGTGTTG	101
Muc5ac	ATCTTTCAGGACCCCTGCTC	ATGGACCACTGGCGTTAGTC	88
Muc6	TTGGCTTAATTGAGTTGGCA	CCCAAAACTTGTTCTCCACA	102
Muc13	CAACTCAGCCTTCTGGTGGT	TACAGGGGTTAGGGTTGCAG	90
Muc20	CAACATCTGCATCCACTGCT	GTCAGCCGTACAAGGAGGAA	82
GSK-3β	GTGGTTACCTTGCTGCCATC	GACCGAGAACCACCTCCTTT	106
Apc	GCCAGGATCCAGCAAATAGA	CATGCCTGCTCTGAGATGAC	97

Table 3-3 qPCR primers

3.4 AB/HID staining

Ileal and colonic segments were fixed in Carnoy fixative (60% ethanol, 10% acetic acid glacial and 30% chloroform) to preserve the mucus layer and transferred in 100% ethanol until paraffin embedding (Matsuo et al., 1997). 4 µm sections were rehydrated and incubated for 24 hours in high iron diamine solution. To prepare high iron diamine (HID) solution 120 mg N,N-dimethyl-meta-phenylenediamine-dihydrochloride (Sigma-Aldrich) and 20 mg N,N-dimethyl-para-phenylenediamine-dihydrochloride (Sigma-Aldrich) were dissolved simultaneously in a Coplin jar in 50 ml of distilled water, then 1,4 ml of freshly prepared 60% ferric chloride solution (Sigma-Aldrich) was added to obtain the final solution. After washing slides were incubated in 1% alcian blue solution in 3% acetic acid (IHC World, NovaUltra). After washing nuclei were counterstained in neutral red solution (0,5% in water, Sigma-Aldrich), deahydrated, cleared and mounted. Alcian

blue in 3% acetic acid stains blue acid mucopolysaccharides and sialomucins, while HID solution reacts with sulfomucins forming black precipitates.

3.5 Bacterial strains

Two *Salmonella* strains were used: *Salmonella enterica* serovar Typhimurium (here referred to as *Salmonella* typhimurium) strain SL1344 ΔAroA (invasive *Salmonella*) and *Salmonella enterica* serovar Typhimurium strain SL1344 ΔAroA InvA^{-} (non-invasive *Salmonella*). ΔaroA mutant is auxotrophic for aromatic amino acids and this confers reduced ability to replicate *in vivo*, while mutation in the invA gene, a component of the SPI-1 encoded type three secretion system (T3SS), results in the inability of the bacterium to invade the host. For infection experiments these strains were grown in Luria Broth with appropriate antibiotics until OD(600nm) ≅ 0,6. Bacteria were administered in 5% sodium carbonate solution and bacterial load of the inoculum controlled by plating.

Clostridium ID4 was isolated in Kenya Honda laboratory (IMS RCAI, Yokohama, Japan) from fecal pellets coming from littermates of our Apc$^{Min/+}$ colony as described in (Atarashi et al., 2013). Briefly, faeces were plated on EG agar plates, a number of colonies were picked and sequenced using 16S universal primers. Similarity to *Clostridium* ID4 was checked on both databases and with the sequence retrieved from our metagenomic analysis, resulting in 99.3% and 98% respectively. Bacteria were cultured in EG broth in anaerobic conditions until OD(600nm) ≅ 0,6 (about 48 hours of culture). Bacteria were frozen in EG 10% glycerol. *C*. ID4 culturing in anaerobic conditions was performed in Marika Falcone Laboratory (DRI Unit, Ospedale San Raffaele, Milan, Italy).

Escherichia coli strain DH5α and a mutant expressing *Yersinia enterocolitica* invasin (*E. coli* DH5α pInv) were grown in Luria Broth with appropriate antibiotics until OD(600nm) ≅ 0,6. Bacterial load of the inoculum was controlled by plating.

3.6 Bacterial spreading

To address bacterial spreading at distal organs wild type littermates and $Apc^{Min/+}$ mice at either 10 or 16 weeks of age were orally administered with 10^9 CFUs of either invasive or non-invasive *Salmonella* in 5% carbonate buffer, or intravenously injected with $5x10^4$ CFUs of invasive *Salmonella*. 72 hours post-infection organs were resected, incubated with gentamycin (50 µg/ml) to eliminate extracellular contamination, digested with collagenase D (1 mg/ml, Roche) and reduced to single-cell suspension. A fixed number of cells was lysed with 0,5% sodium deoxycholate and plated on TB-agar plates in duplicate.

3.7 *Salmonella* vaccination and administration

In vaccination experiments littermate and $Apc^{Min/+}$ mice were orally administered with 10^9 CFUs of invasive *Salmonella* in carbonate buffer every other day for 3 challenges. Serum and fecal samples were taken weekly for the 5 weeks following vaccination and analysed for the presence of *Salmonella*-specific IgG and IgA with ELISA assay as described in paragraph 3.8.

To test weather bacterial challenges induced tumorigenesis littermate and $Apc^{Min/+}$ mice were administered from the 8^{th} week of life with 10^9 CFUs of invasive *Salmonella* in carbonate buffer every other week for four total challenges. *Salmonella* was either administered alone or 24 hours after treatment with streptomycin (20 mg/mouse) as previously described (Barthel et al., 2003). Serum and faeces were sampled every other week and the development of *Salmonella*-specific IgA and IgG assessed by ELISA assay as described in paragraph 3.8 and 3.9. Mice were sacrificed at the 16^{th} week of age and tumor multiplicity in the small intestine and colon assessed.

3.8 ELISA for total IgA and *Salmonella*-specific IgA and IgG

For IgA and IgG detection blood was sampled via tail vein bleeding and serum stored at -80°. Fecal pellets were homogenised in 1 ml/0,1 g faeces of 0,1 mg/ml SBTI (Trypsin

inhibitor from *Glicine max*, Sigma Aldrich), centrifuged at 13000 rpm and supernatants stored at -80°C. Sera were tested at 1:100 and faeces at 1:10 dilution in duplicate.

For total IgA detection plates were coated with 0,2 µg/well purified anti-mouse IgA (clone C10-3, BD Biosciences) and blocked in PBS 0,5% BSA. Samples were diluted in PBS 0,05% Tween 20 and incubated at 37°C for 2 hours. After washing plates were incubated with 0,2 µg/well biotin anti-mouse IgA (clone C10-1, BD Biosciences) for 1 hour at room temperature and then with Streptavidin-HRP (R&D systems) for 30 minutes. Plates were developed with the colorimetric substrate TMB (Sigma Aldrich) and the reaction is stopped with sulphuric acid 2N. Absorbance signal is red at 450 nm.

For the detection of *Salmonella*-specific immunoglobulins plates were coated with *Salmonella* lysate (10^5 CFU/well) and blocked in PBS 1% BSA 5% sucrose. After washing plates are incubated with the samples as previously described. After washing plates were incubated with anti-mouse IgA-HRP antibody (diluted 1:1000, cat 1040-05, SouthernBiotec) for IgA and with anti-mouse IgG-HRP (diluted 1:1000, cat. 1030-05, SouthernBiotech) for IgG for 1 hour at room temperature. Plates were then washed and developed as previously described.

3.9 ALT detection

Alanine transaminases were detected in sera of infected and control mice with ALT Microwell Autom kit (Sentinel Diagnostics) following the manufacturer's instructions. Samples were diluted 1:10 in PBS. Transaminases are involved in protein metabolism and are present in different organs, but their detection in the serum is associated with liver cell damage. The kit is based on an enzymatic reaction cascade resulting in the development of a colorimetric signal detected at 550 nm.

3.10 AOM/DSS

C57BL/6J females aged 8 weeks were treated with azoxymethane (AOM) 10 mg/Kg of body weight by intraperitoneal injection. After 7 days control groups received respectively nothing and N-acetyl cysteine (NAC) 1% w/v in drinking water (groups

AOM/H_2O and AOM/NAC). Two cohorts received DSS 2% w/v in drinking water for 7 days (AOM/DSS and AOM/DSS NAC). Both groups were allowed for recovery for 14 days but while group AOM/DSS received water upon DSS stop, group AOM/DSS NAC received NAC 1%. This schedule was repeated for 3 cycles. Body weight and survival were assessed periodically. Mice were sacrificed at day 70 of treatment. Colons were fixed in formalin-buffered solution and paraffin-embedded.

For histopathological examination single 4 μm-thick haematoxylin and eosin stained swiss roll sections were evaluated under a light microscope. The extent of inflammatory changes was evaluated according to the score proposed by (Cooper et al., 1993). Histological scoring system used to evaluate colitis grade is described in Table 3-4. Additionally the number of ulcers and neoplastic lesions was recorded. Classification of proliferative lesions has been performed according to (Boivin et al., 2003).

Grade of colitis	Description
0	Normal colonic mucosa
1	Minimal inflammation restricted to the mucosa and only slightly expanding the lamina propria among crypts or between crypts and *muscularis mucosae*.
2	Mild to moderate (usually focal) inflammation in the mucosa with shortening of the crypts and occasionally mildly extending to the submucosa (usually associated with small ulcers)
3	Moderate to severe (multifocal to diffuse) inflammation involving the mucosa with shortening or loss of the crypts and moderately to markedly extending into the submucosa and occasionally into the *tunica muscularis* (usually associated with large ulcers)
4	Severe transmural inflammation involving the mucosa, submucosa, tunica muscularis, and subserosa (usually associated with diffuse ulceration).

Table 3-4 Colitis histological scoring system

3.11 DNA extraction from fecal samples

DNA from faecal pellets and mucus scraped from the small intestine and colon was extracted with G NOME DNA isolation kit (MP) following a published protocol (Furet et al., 2009). Briefly, fecal pellets were stored at -80°C until the extraction. Fecal pellets were homogenised in 550 µl Cell Suspension Solution (G NOME DNA Kit). After addition of 50 µl RNase Mixx (G NOME DNA Kit) and 100 µl Cell Lysis/Denaturing Solution (G NOME DNA Kit) samples were incubated at 55°C under shaking for 30 minutes. After adding 25 µl Protease Mixx (G NOME DNA Kit) samples were incubated for further 2 hours at 55°C under shaking. Samples then undergo mechanical disruption of bacterial cells with 0,1 mm glass beads (BioSpec Products) in FastPrep®-24 homogenizer (MP Biomedicals). Lysates were retrieved. Glass beads are washed three times with 400 µl of TENP buffer (50 mM Tris pH 8, 20 mM EDTA pH 8, 100 mM NaCl, 1% PVPP). Supernatants are pooled with the original lysate and precipitated with isopropanol. DNA pellet was resuspended in 400 µl water and incubated with 100 µl of Salt.Out Mixture (G NOME DNA Kit) to remove impurities. Samples are then precipitated in 100% ethanol and DNA pellet washed with 70% ethanol. DNA pellets are dried and resuspended in water.

3.12 Metagenomic analysis

Faecal material was sampled from littermate and $Apc^{Min/+}$ mice at 4, 8, 12 and 16 weeks of age and DNA purified as described in paragraph 3.11.

The subsequent steps involving sample preparation for sequencing, the sequencing and bioinformatics analysis were conducted in the laboratory of Prof. Graziano Pesole (Institute of Biomembranes and Bioenergetics, National Research Council -CNR-, Bari, Italy) and will be here summarized.

V5-V6 hypervariable regions of bacterial 16S rDNA were amplified and processed with a modified version of the Nextera protocol. Metagenomic libraries obtained were sequenced with MiSeq Illumina platform with 2x250 paired-end approach. Samples of the four time points were sequenced in four different runs. Quality check with the tool

Trim Galore! removed low quality regions (Phred quality score cut-off = 25) and sequences shorter than 50 bp. Table 3-5 summarizes the number of total reads per run and the mean number of reads per sample.

	n° reads/run	n° reads/sample mean ± SD	Mean read length ± SD (bp)
4 weeks	22359242	678791 ± 275306,616	249,01 ± 18,72
8 weeks	24795998	590270 ± 158089,465	249,23 ± 17,07
12 weeks	24585612	604681 ± 186225,132	248,83 ± 19,60
16 weeks	23867080	674281 ± 217118,955	248,73 ± 19,96

Table 3-5 Sequencing data details. Every time point was sequenced in a single run. For each run the total number of reads, the mean number of reads per sample (mean ± SD) and the mean read length (length ± SD) is shown.

Forward and reverse reads were overlapped creating consensus sequences. Reads without partner overlapping sequence were excluded from the analysis. Consensus sequences were aligned with the Bowtie 2 tool to the Ribosomal Database Project II (RDP II, release 10.29), a database of aligned and annotated rRNA gene sequences (Cole et al., 2003). Mapping data were filtered with a 97% similarity cut-off and 70% of coverage of the aligned sequence. Taxonomic alignment of matching sequences was performed with TANGO tool. Assigned genera were filtered considering as present only the ones for which at least 5 reads per samples were present (Table 3-6). Finally, the number of reads assigned to each taxon was normalized to the total number of taxon assigned reads.

	Pre-filtering	Post-filtering
4 weeks	631	74
8 weeks	551	61
12 weeks	586	67
16 weeks	652	81

Table 3-6 Taxonomic data relative to genera pre- and post-filtering.

3.13 *Clostridium* ID4 qPCR assay

Primers specific to *Clostridium* ID4 were designed in the 16S rDNA in an area of minimal overlap to other *Clostridium* species. To exclude detection of most known Clostridial species primers were tested with *in silico* PCR tool (http://insilico.ehu.es/PCR/).

To detect *C*. ID4 in the faeces or mucus with qPCR assay DNA was extracted as described in paragraph 3.11. 10 ng of cDNA were amplified with *C*. ID4 specific primers (final concentration 1 µM) using Sybr Green Master Mix (Life Technologies). *C*. ID4 abundance was normalized to primers amplifying eubacterial 16S (EUB) (Salzman et al., 2010). Primer sequences are listed in Table 3-7.

Species	Forward primer (5'→3')	Reverse primer (5'→3')	Amplicon (bp)
C. ID4 16S	CCGGGAATACGCTCTGGAAA	GCCAACCAACTAATGCACCG	123
EUB 16S	GGTGAATACGTTCCCGG	TACGGCTACCTTGTTACGACTT	172

Table 3-7 Primers used for qPCR assay on bacterial DNA

3.14 *Clostridium* ID4 administration to GF and Apc$^{Min/+}$ mice

Experiments of gnotobiotic colonization of germ-free mice with *Clostridium* ID4 were performed in Kenya Honda laboratory (IMS RCAI, Yokohama, Japan). Briefly 5 ICR male mice were administered with 250 µl of *C*. ID4 culture. After 5 weeks cells were isolated from the lamina propria of the small intestine and colon and the abundance of

Tregs (percentage of FoxP3+ cells in the CD4+ population), IL17-producing cells (percentage of IL17+ cells in the CD4+ or CD8+ population) and IFNγ-producing cells (percentage of IFNγ+ cells in the CD4+ or CD8+ population) was evaluated by flow cytometry. *C*. ID4 gnotobiotic mice were compared with both germ-free and SPF mice.

To evaluate the effect of exogenous *C*. ID4 administration WT and $Apc^{Min/+}$ mice were orally administered from 6 to 12 weeks of age twice a week with frozen *C*. ID4 stocks equivalent to 250 µl of culture (see paragraph 3.5) according to the protocol used in (Atarashi et al., 2013). Mice were weekly sampled for faeces to check *C*. ID4 abundance as described in paragraphs 3.11 and 3.13. At 12 weeks of age mice were sacrificed, tumor multiplicity in the small intestine and colon assessed. Neutrophil and inflammatory monocyte abundances in circulating blood and Treg, Th1 and Th17 abundances in small intestinal and colonic lamina propria assessed as described in paragraphs 3.2 and 3.15 respectively.

3.15 FACS on LP cells

To analyse T cell populations in the lamina propria of WT and $Apc^{Min/+}$ mice small intestines and colons were incubated with PBS 5% FCS 1,5 mM EDTA 1mM DTT with magnetic stirrer at 37°c for 15 minutes to remove IECs. Intestinal pieces were then mechanically disrupted in RPMI 5% FCS with 2 consecutive cycles of gentleMACS Dissociator (Miltenyi Biotec) program m_intestine_01.

The surface and intranuclear staining for Treg detection cells were incubated with anti CD16/CD32 (clone 93, eBioscience) to block Fc receptors. For membrane staining cells were incubated with CD45.2 PerCP-Cy5.5 (clone 104, eBioscience), CD3 PE-Cy7 (clone SK7, BD Biosciences), CD4 FITC (clone GK1.5, BD Biosciences), CD25 APC-Cy7 (Clone PC61, BioLegend) and Fixable Viability Dye eFluor® 450 (eBioscience) to exclude dead cells. Cells were then incubated with Fixation/Permeabilization buffer (eBioscience) for 1 hour at 4°C and then stained with FoxP3 PE (clone FJK-16s, eBioscience) and Helios Alexa-Fluor 647 (clone 22F6, eBioscience).

For Th1 and Th17 detection cells were re-stimulated *in vitro* with PMA (50 ng/ml, Sigma Aldrich), ionomycin (500 ng/ml, Sigma Aldrich) and GolgiStop (4 µl/6ml, BD Biosciences) for 4 hours at 37°C. Membrane and intracellular staining were performed as previously described. For surface markers cells were stained with CD45.2 PerCP-Cy5.5 (clone 104, eBioscience), CD3 PE-Cy7 (clone SK7, BD Biosciences), CD4 FITC (clone GK1.5, BD Biosciences), CD8 APC eFluor 780 (clone 53-6.7, eBioscience) and Fixable Viability Dye eFluor® 450 (eBioscience) to exclude dead cells. Intracellular cytokines were stained with IL17 PE (clone TC11-18H10, BD Biosciences) and IFNγ APC (clone XMG1.2, BD Biosciences).

Samples were acquired at FACSCantoII (BD Biosciences).

3.16 miRNA analysis

To compare miRNA profiles in the intestines of WT and $Apc^{Min/+}$ mice RNA isolated from single ileal crypts was analysed with murine miRNA expression cards. Sample preparation, miRNA profiling and the subsequent analysis were performed in Prof. Silvano Bosari laboratory (Department of Patophisiology and Transplantation, University of Milan, Italy). Briefly, RNA was extracted from 8 16 weeks old WT and 8 $Apc^{Min/+}$ formalin-fixed paraffin embedded ileal segments. Laser-assisted microdissection allowed recovery of single crypts from the WT tissue and both normal (N) and dysplastic (D) crypts from $Apc^{Min/+}$ tissue. RNA was retro-transcribed and amplified (Megaplex RT and PreAmp Primers Rodent Pools A+B, Life Technologies). cDNA obtained from all the WT, N and D crypts were pooled and miRNA cards were performed (TaqMan® Array Rodent MicroRNA A+B Cards Set, Life Technologies). Relative quantification was calculated relative to the three reference snoRNAs (U6, sno202 and sno135) with DataAssist Software (Life Technologies). BRB Array Tools was used for supervised analysis to compare WT vs. N and N vs. D profiles using a cut-off of 10 fold on $\log_2$ data.

3.17 *Ex-vivo* infection of murine colonic mucosa

To address whether bacterial stimulation could alter *apc* gene expression we stimulated colonic explants with an *ex-vivo* organ culture model developed in our laboratory (Tsilingiri et al., 2012; Tsilingiri et al., 2013). Briefly, colonic tissue from WT and $Apc^{Min/+}$ mice at 8 weeks of age was incubated in gentamicin (50 μg/ml) to remove contaminants. The tissue was then placed on a grid and a cylinder (diameter 6 mm) sealed on it to allow polarized stimulation of the mucosa as shown in Figure 3-2. Samples were infected with 10^2 CFUs in a volume of 50 μl of ΔaroA invA *Salmonella* thyphimurium, ΔaroA *Salmonella* thyphimurium, *E. coli* DH5α or *E. coli* DH5α pInv. After 2 hour at 37°C the infection medium was removed from the cylinder, gentamycin was added to the culture medium (50 μg/ml) and samples were incubated for 24 hours in 100% O_2 atmosphere. RNA extraction was performed and *apc* gene expression was evaluated as described in paragraph 3.3.

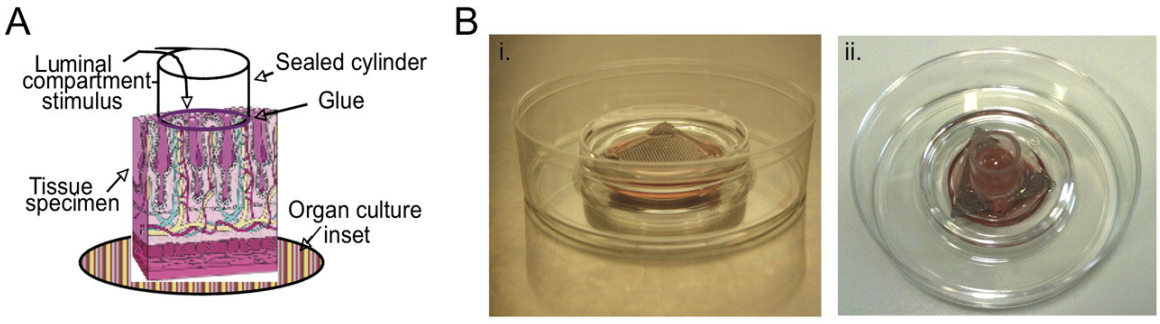

Figure 3-2 *Ex-vivo* organ culture method. A piece of intestinal mucosa is placed on a grid and a cylinder is sealed on the luminal side of the tissue. The stimulus is placed into the cylinder so that the tissue is stimulated from the luminal side as happens in physiological conditions. Adapted from (Tsilingiri et al., 2012).

3.18 Statistical analysis

Results are represented as mean ± SEM. Statistical significance was evaluated with Student's *t* test were evaluated using GraphPad Prism software.

*p<0.05, **p<0.01, ***p<0.001.

4 RESULTS

4.1 $Apc^{Min/+}$ mice characterization

4.1.1 $Apc^{Min/+}$ mice develop adenomas in the small intestine

The $Apc^{Min/+}$ mouse is a model of APC driven intestinal tumorigenesis. It is characterized by the min mutation that results in a truncated and non-functional APC protein product (Moser et al., 1990). Truncated APC results in a non functional β-catenin degradation complex and β-catenin is free to migrate to the nucleus and activate transcriptional programs involved in cell proliferation and survival (Anastas and Moon, 2013). The min mutation is in heterozygosity and the loss of the WT allele leads to the progressive accumulation of adenomatous polyps in the SI and colon, with greater incidence in the distal portion of the SI (Moser et al., 1990).

A characterization of $Apc^{Min/+}$ mice in our housing conditions and experimental setting shows that, as described in literature, mice start accumulating polyps in the small intestine from the eighth week of life onwards, reaching a plateau at about 14 weeks of age (Figure 4-1). Polyp number is maximal in the distal part of the SI (e.g. in the ileum) while it is sporadic in the colon (Figure 4-2). Figure 4-3 shows a representative picture of the different tracts of the small intestine and colon of an $Apc^{Min/+}$ mouse at 16 weeks of age showing the appearance of the polyps.

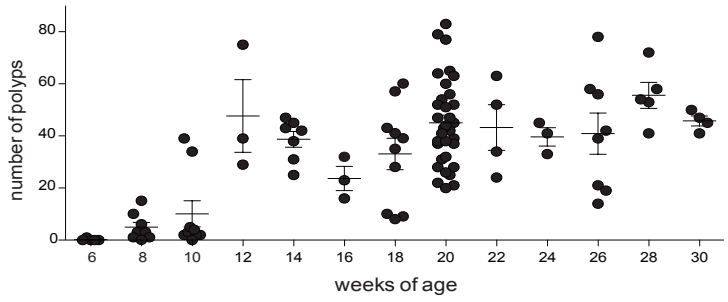

Figure 4-1 Tumor multiplycity in the small intestine of $Apc^{Min/+}$ mice. $Apc^{Min/+}$ mice were sacrificed at different weeks of age and tumor number in the SI was assessed.

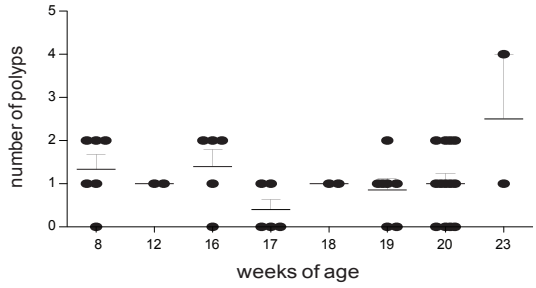

Figure 4-2 Tumor multiplycity in the colon of Apc$^{Min/+}$ mice. Apc$^{Min/+}$ mice were sacrificed at different weeks of age and tumor number in the colon was assessed.

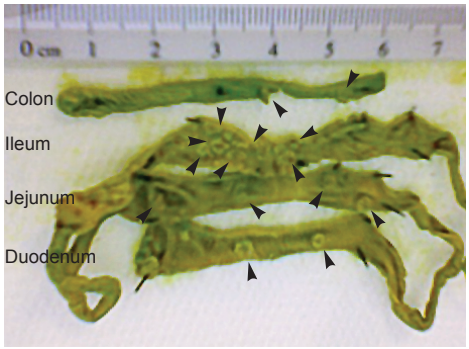

Figure 4-3 Polyp appearance in the intestine of Apc$^{Min/+}$ mice. Representative picture of a longitudinally opened intestine and colon to show the appearance of the adenomas. Intestines were fixed with Hollandes fixative and longitudinally opened. The picture shows from top to bottom colon, ileum, jejunum and duodenum. Arrows point to polyps.

4.1.2 Apc$^{Min/+}$ mice develop hematopoietic disorders

It is reported that Apc$^{Min/+}$ mice have hematological alterations characterized by thymic atrophy, B and T cell depletion and reduction of NK cell abundance while myelopoiesis is enhanced if compared to WT mice (Coletta et al., 2004)(Guglietta S., unpublished).

An analysis of the principal immune cell populations by FACS staining showed that Apc$^{Min/+}$ mice had comparable T (CD4+ and CD8+) and B (B220+ CD19+) cell abundance in circulating blood compared to littermates until 16 weeks of age (Figure 4-4). However at later time points we observed a reduction in the mature B cell population. More interestingly we observed a dramatic decrease in neutrophils (Ly6G+

CD11b+) both in circulating blood and in mesenteric lymph nodes as previously reported (Figure 4-4, Figure 4-5) (Coletta et al., 2004)(Guglietta S., unpublished).

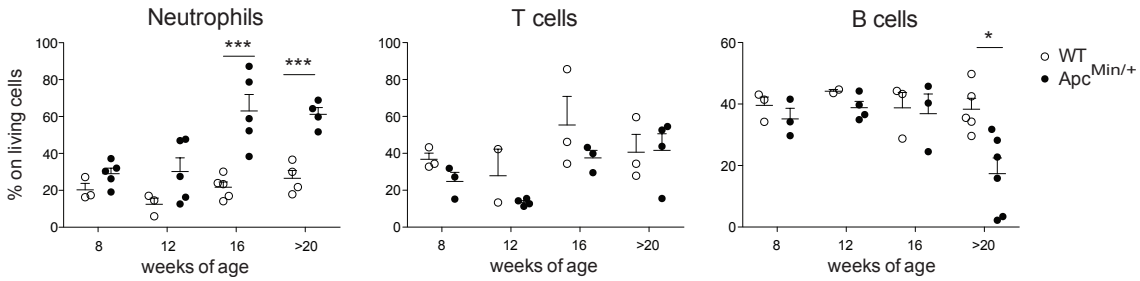

Figure 4-4 Neutrophil, T and B cell abundance in the peripheral blood of WT and Apc$^{Min/+}$ mice. FACS analysis showing the abundance of neutrophils (Ly6G+ CD11b+), T cells (CD4+ and CD8+) and B cells (B220+ CD19+) in the peripheral blood of WT and Apc$^{Min/+}$ mice at different weeks of age. Data are shown as percentages of the total population. Graphs show mean ± SEM. n=3/6. * p<0,05; *** p<0,001.

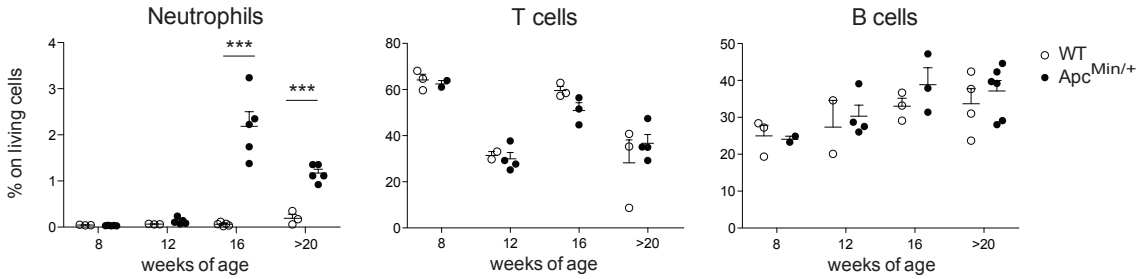

Figure 4-5 Neutrophil, T and B cell abundance in the mesenteric lymph node of WT and Apc$^{Min/+}$ mice. FACS analysis showing the abundance of neutrophils (Ly6G+ CD11b+), T cells (CD4+ and CD8+) and B cells (B220+ CD19+) in the mLNs of WT and Apc$^{Min/+}$ mice at different weeks of age. Data are shown as percentages of the total population. Graphs show mean ± SEM. n=3/6. *** p<0,001.

4.2 The mucus barrier is altered in intestinal tumorigenesis

4.2.1 Mucin expression is altered in the early phases of tumorigenesis in Apc$^{Min/+}$ mice

Mucin expression and post-traslational modification status is altered in pathological conditions of the intestine, such as IBD and hCRC (Sheng et al., 2012). To address

whether in murine models of intestinal tumorigenesis as for the human disease the mucus barrier is altered we analysed mucin expression in $Apc^{Min/+}$ intestines.

RNA from intestines of wild type mice, normal intestinal tissue and single polyps of $Apc^{Min/+}$ mice was analysed by RT-qPCR assay for a panel of mucins. Intestinal samples were taken from mice at both an early (10 weeks of age) and very late (23 weeks of age) stage of tumor progression. Results about the ileum show that mucin expression profile in the tumor of $Apc^{Min/+}$ mice is altered both at 10 and 23 weeks of age (Figure 4-6). In particular, mucins that are normally not expressed or expressed at very low levels in the intestine such as Muc1, Muc5ac, Muc6 and Muc20 increase in tumors while the expression of mucins that are constitutively expressed in the gut such as Muc3 and Muc13 decreases (Figure 4-6). Interestingly Muc1 expression is altered already in the normal tissue of $Apc^{Min/+}$ mice while Muc2 expression is only slightly reduced in the tumors of $Apc^{Min/+}$ mice at 23 weeks of age (Figure 4-6).

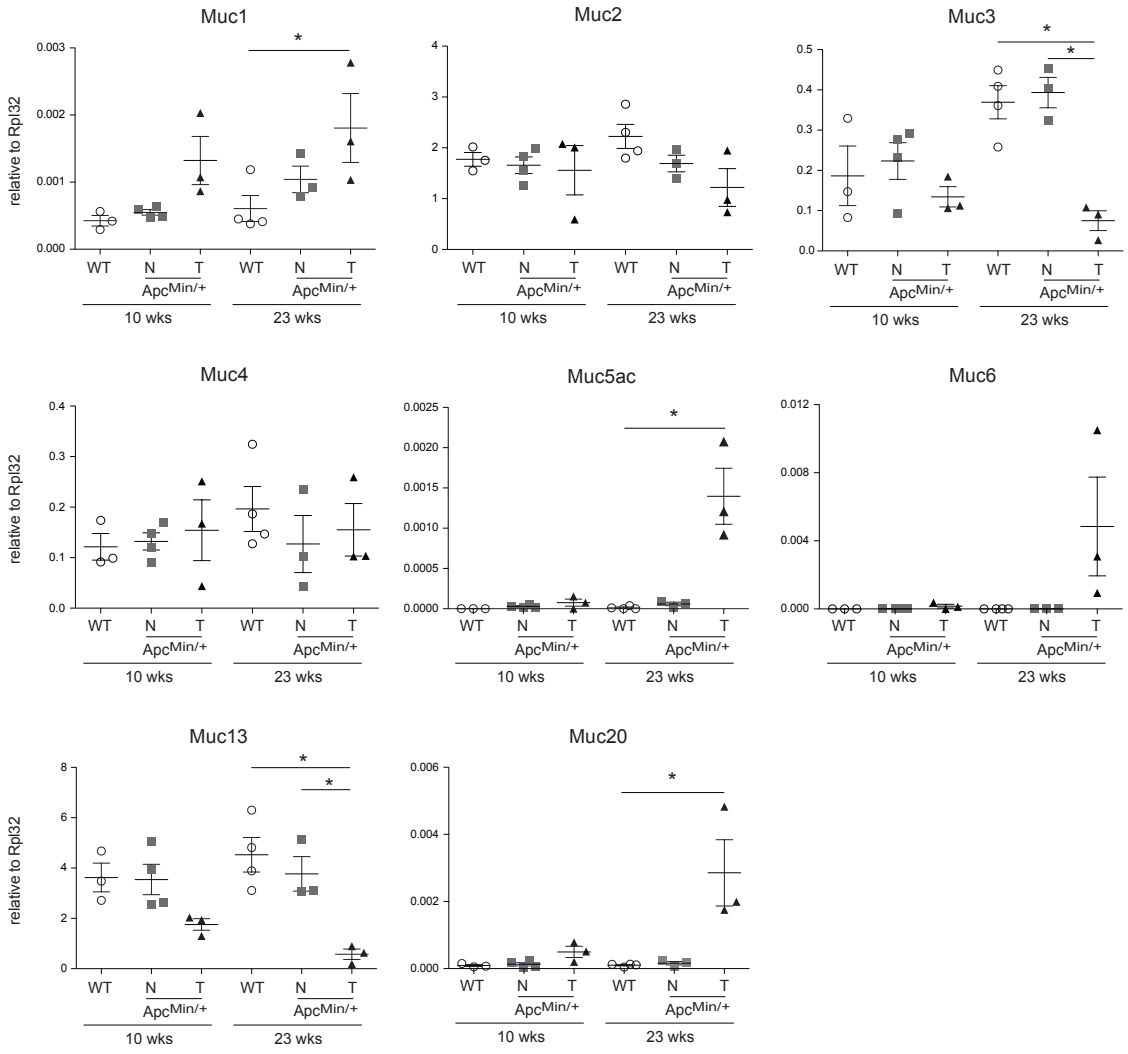

Figure 4-6 Mucin expression profile is altered in the ileum of Apc$^{Min/+}$ mice. Ileal tissue from WT (WT) and both normal ileal tissue (N) and tumors (T) from Apc$^{Min/+}$ ilei at 10 and 23 weeks of age was analysed by RT-qPCR for the expression of Muc1, Muc2, Muc3, Muc4, Muc5ac, Muc6, Muc13 and Muc20. Expression levels are normalized to Rpl32. Graphs show mean ± SEM. n=3/4. * p<0,05.

Altered mucin expression was evident also when single crypts isolated by laser-assisted microdissection were analysed (Figure 4-7). Dysplastic crypts isolated from Apc$^{Min/+}$ ilei showed increased expression of Muc5ac and Muc20 compared to wild type crypts and normal crypts from Apc$^{Min/+}$ ileum, while Muc3 and Muc13 were reduced (Figure 4-7). Taken together these data show that mucin expression level is altered in the polyps and dysplastic crypts in the Apc$^{Min/+}$ ileum.

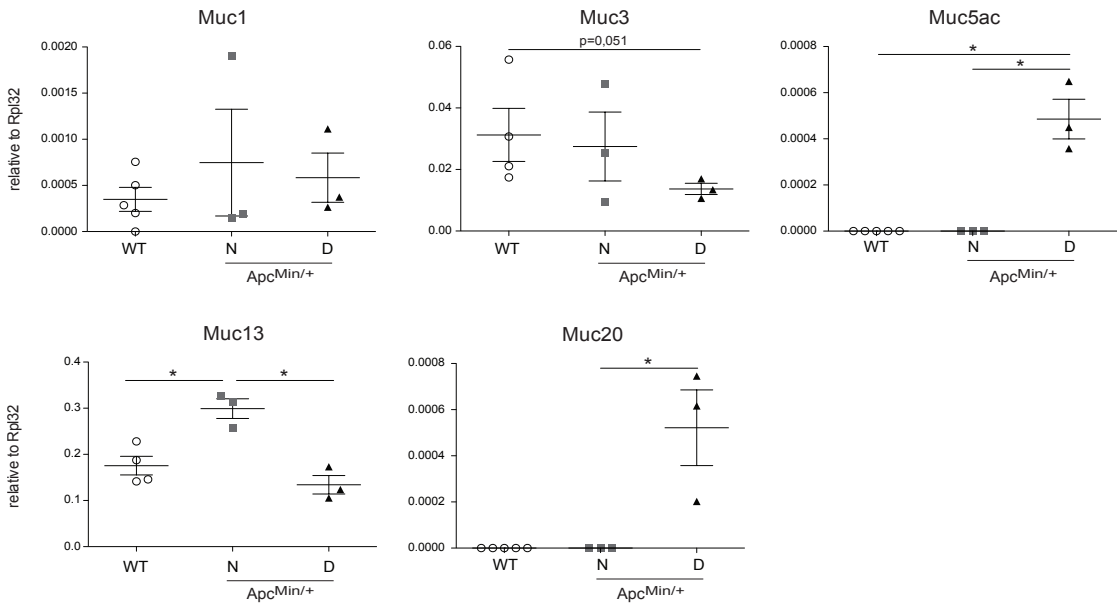

Figure 4-7 Mucin expression profile is altered in the dysplastic crypts of Apc$^{Min/+}$ mice. Single crypts from WT (WT) ileum and both normal (N) and dysplastic (D) crypts from Apc$^{Min/+}$ ileum at 16 weeks of age were analysed by RT-qPCR for the expression of Muc1, Muc3, Muc5ac, Muc13 and Muc20. Expression levels are normalized to Rpl32. Graphs show mean ± SEM. n=3/4. * p<0,05.

On the contrary mucin expression profile in the colon of Apc$^{Min/+}$ mice showed only minor alterations (Figure 4-8). As colon tumorigenesis is a sporadic event in the Apc$^{Min/+}$ model data regarding colonic tumors at 10 and 23 weeks of age are represented together. In both the normal colonic tissue and polyps of Apc$^{Min/+}$ mice we observed a trend towards a reduction in Muc2, Muc3 and Muc13 (Figure 4-8). The reduced expression of these gut specific mucins points to an alteration in the mucus barrier in the Apc$^{Min/+}$ also at the colonic level.

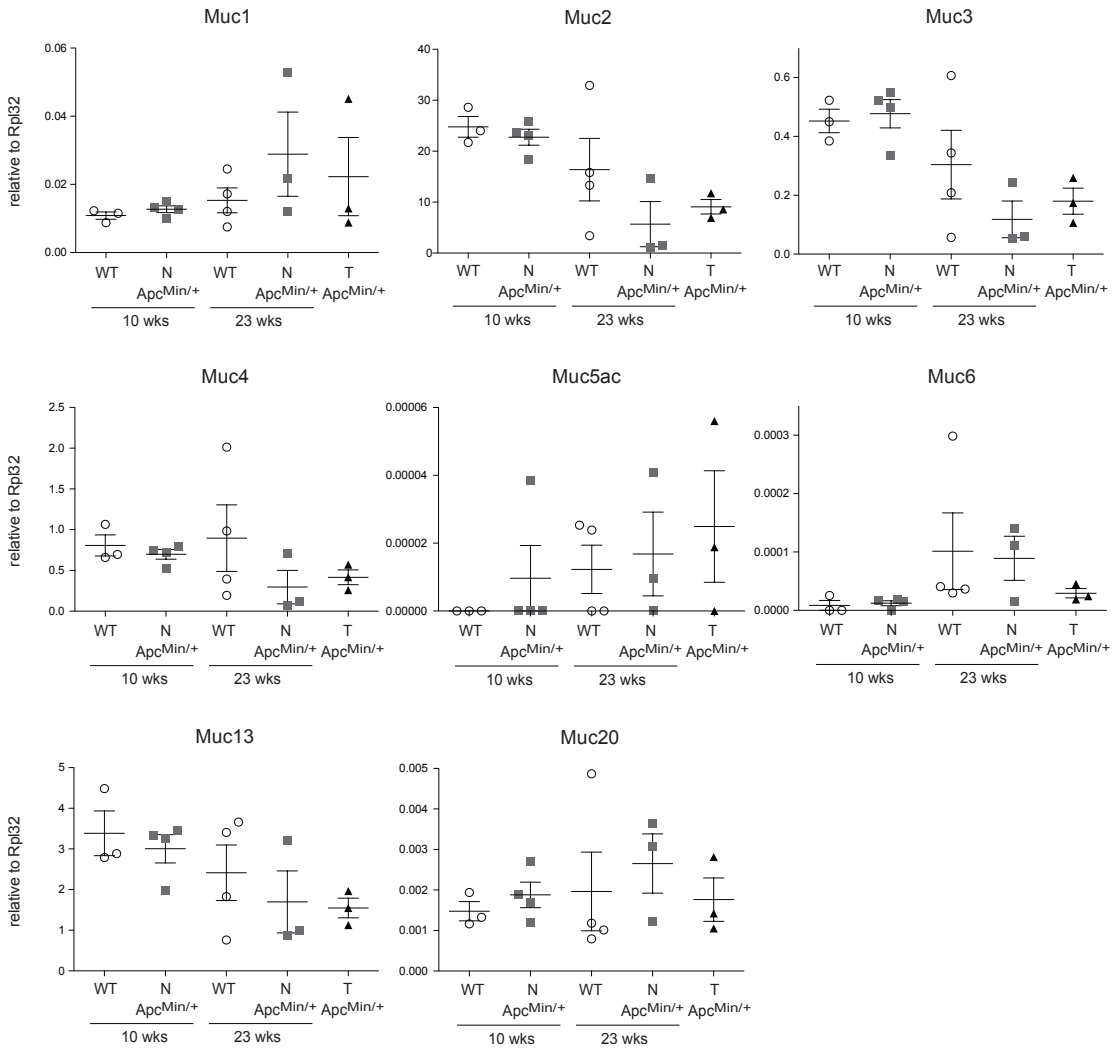

Figure 4-8 Mucin expression profile is only partially altered in the colon of Apc$^{Min/+}$ mice. Colonic tissue from WT (WT) and both normal colon tissue (N) and tumors (T) from Apc$^{Min/+}$ ilei at 10 and 23 weeks of age was analysed by RT-qPCR for the expression of Muc1, Muc2, Muc3, Muc4, Muc5ac, Muc6, Muc13 and Muc20. Expression levels are normalized to Rpl32. Data about tumors from 10 and 23 week-old Apc$^{Min/+}$ mice are represented together. Graphs show mean ± SEM. n=3/4. * p<0,05.

Since we observed increased expression of Muc1 in ileal polyps and dysplastic crypts of Apc$^{Min/+}$ mice and Muc1 intracellular domain interacts with GSK-3β (Li et al., 1998) we tested whether its transcription was altered in our model. We found that GSK-3β expression is reduced in Apc$^{Min/+}$ polyps (Figure 4-9). As GSK-3β-mediated MUC1 phosphorylation disrupts its interaction with β-catenin (Li et al., 1998), the parallel increase in Muc1 and decrease in GSK-3β expression in the polyps of Apc$^{Min/+}$ mice could contribute in the alteration of β-catenin cellular pools and thus deregulation of the Wnt signalling pathway.

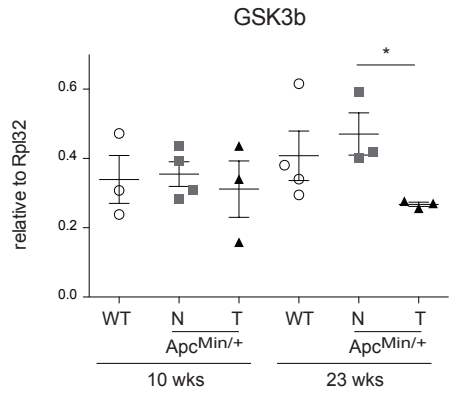

Figure 4-9 GSK-3β expression is reduced in the polyps of Apc$^{Min/+}$ mice. Ileal tissue from WT (WT) and both normal ileal tissue (N) and tumors (T) from Apc$^{Min/+}$ ilei at 10 and 23 weeks of age was analysed by RT-qPCR for the expression of GSK-3β. Expression levels are normalized to Rpl32. Graph shows mean ± SEM.n=3/4. * p<0,05.

4.2.2 Mucus sulphonation is not altered in Apc$^{Min/+}$ intestine

Mucins are highly glycosylated proteins (McGuckin et al., 2011) and their glycosylation pattern is often altered in pathological conditions of the intestine, including CRC (Sheng et al., 2012). Sulphonation is an important post-traslational modification of intestinal mucins and is fundamental in the maintenance of proper barrier functions including resistance to colitis and enteric infection (Dawson et al., 2009). We therefore assessed if mucus sulphonation in addition to mucin expression is altered in the Apc$^{Min/+}$ model. Sulphonation was visualized with high iron diamine (HID) coupled alcian blue (AB) staining on ileal and colonic sections.

In the small intestine of WT and Apc$^{Min/+}$ mice we barely detected any sulphonation signal (Figure 4-10), with no differences between WT and Apc$^{Min/+}$ mice in both the proximal and distal portions of the small intestine. In the colon we detected a sulphonation signal that was more intense in the distal part of the colon (Figure 4-11). However also in the colon we did not detect any difference between wild type and Apc$^{Min/+}$ mice. We can hence conclude that sulphonation is not altered in the Apc$^{Min/+}$ model.

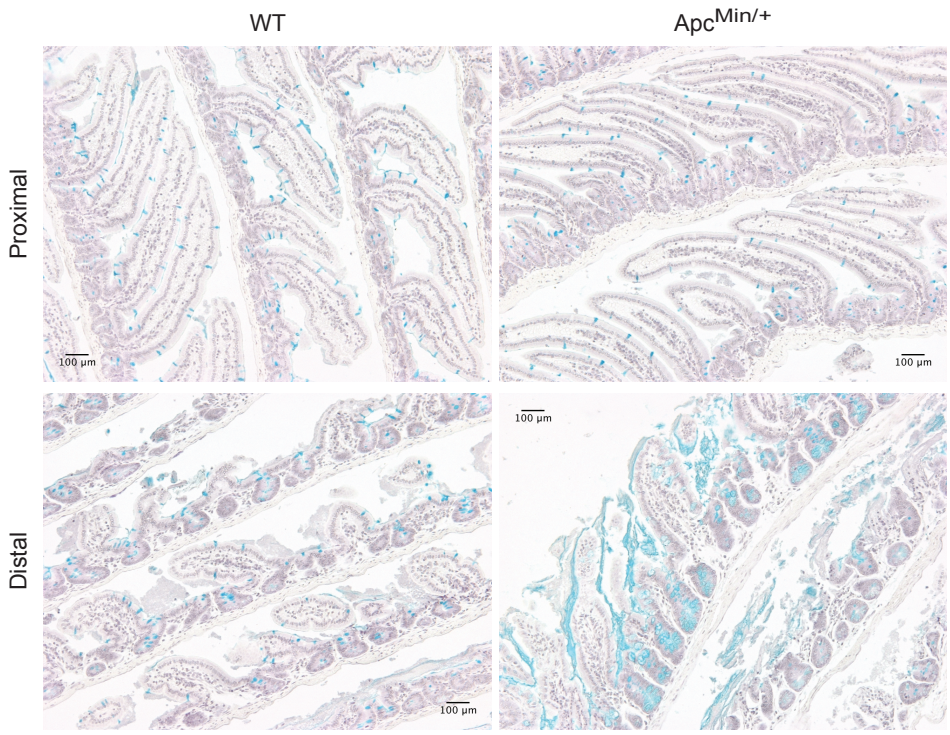

Figure 4-10 Mucus sulphonation is not altered in the small intestine of Apc$^{Min/+}$mice. 4 µm small intestinal sections from WT and Apc$^{Min/+}$ mice were stained with AB/HID. Representative pictures from proximal and distal SI are shown. Blue: sialomucins, black: sulphomucins.

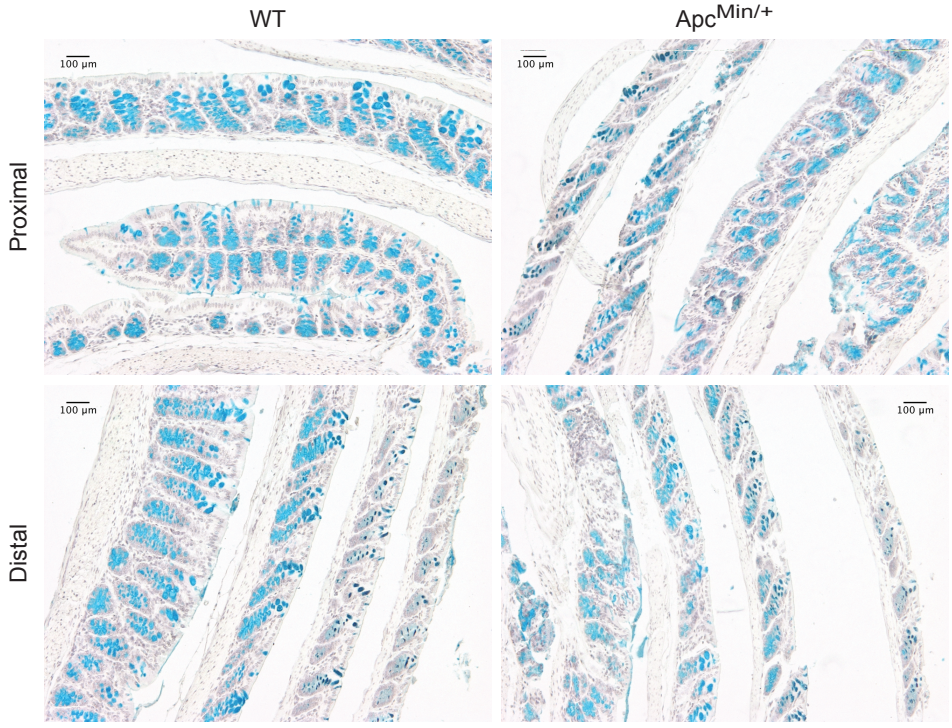

Figure 4-11 Mucus sulphonation is not altered in the colon of Apc$^{Min/+}$ mice. 4 µm colon sections from WT and Apc$^{Min/+}$ mice were stained with AB/HID. Representative pictures from proximal and distal colon are shown. Blue: sialomucins, black: sulphomucins.

4.3 Intestinal permeability is altered in Apc$^{Min/+}$ mice

4.3.1 *Salmonella* preferentially localizes in the mLN, small intestine and polyps of Apc$^{Min/+}$ mice

It has been reported that intestinal adenomas lead to altered intestinal permeability (Grivennikov et al., 2012). Having observed altered mucus composition in tumor bearing mice we addressed whether this altered barrier properties resulted in altered bacterial penetration. To this extent we employed *Salmonella* typhimurium, a microorganism that is able to interact and digest the mucus (McCormick et al., 1988; Nevola et al., 1987; Vimal et al., 2000) and thus could better highlight differences due to alterations in the mucus barrier. We orally administered ΔaroA *S.* typhimurium to WT and Apc$^{Min/+}$ mice at 10 weeks of age and bacterial dissemination to organs was assessed at 72 hours post infection (hpi) (Figure 4-12). Bacterial burden was higher in the mLNs of Apc$^{Min/+}$ mice when compared to WT littermates, while there was no difference in the CFU titre recovered from the spleen (Figure 4-12). Consistently we did also recover more CFUs from the small intestine of Apc$^{Min/+}$ mice than from littermates (Figure 4-12). In this experiment mice were at an early stage of tumorigenesis and it was possible to analyse bacterial penetrance only in the macroscopically healthy small intestinal mucosa of Apc$^{Min/+}$ mice.

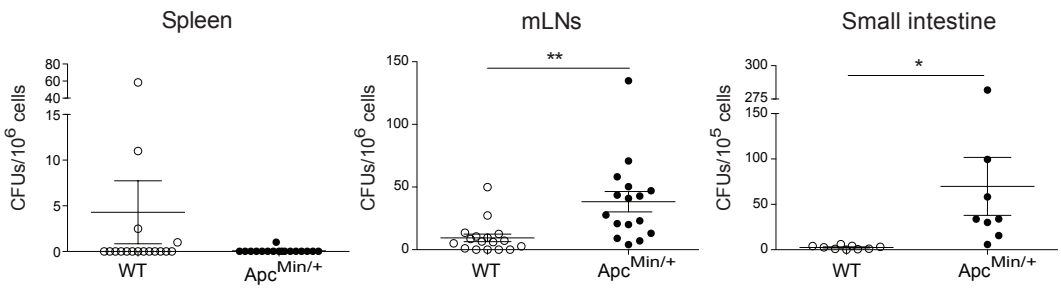

Figure 4-12 Apc$^{Min/+}$ mice have increased bacterial permeability at early stages of tumorigenesis. 10 weeks old WT and Apc$^{Min/+}$ mice were orally infected with 10^9 CFUs of ΔaroA *S.* typhimurium. CFU load in the spleen, mLN and SI was addressed at 72 hpi. Graphs show mean ± SEM. n=8/16. * p<0,05, ** p<0,01.

We did also address bacterial penetrance at a later stage of tumor development to test whether intestinal permeability was altered also at the level of the polyps. We infected littermates and Apc$^{Min/+}$ mice at 16 weeks of age with ΔaroA *S.* typhimurium and analysed bacterial load at 72 hpi (Figure 4-13). Again bacterial spreading to the mLNs was higher in Apc$^{Min/+}$ Mice compared to littermates and we recovered higher bacterial loads from the polyp-free intestinal tissue of Apc$^{Min/+}$ mice. Interestingly we observed significantly higher bacterial titre both in the tumors and in the normal ileum of Apc$^{Min/+}$ mice when compared to wild type ilei. In neither cases we observed differences in CFU numbers recovered from the spleens (Figure 4-13). These data highlight a differential intestinal permeability in Apc$^{Min/+}$ mice upon challenge with invasive *Salmonella* at both early and late stages of tumor development.

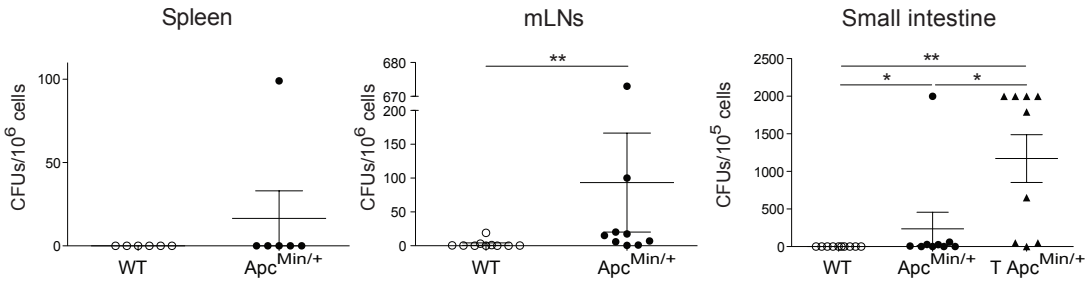

Figure 4-13 Apc$^{Min/+}$ mice have increased bacterial permeability at late stages of tumorigenesis. 16 weeks old WT and Apc$^{Min/+}$ mice were orally infected with 10^9 CFUs of ΔaroA *S.* typhimurium. CFU load in the spleen, mLN and SI was addressed at 72 hpi. Graphs show mean ± SEM. n=6/9. * p<0,05, ** p<0,01.

To rule out the possibility that the difference in bacterial spreading observed was due to the invasiveness of the *Salmonella* strain used we performed the bacterial spreading assay using the non-invasive invA ΔaroA *S.* typhimurium strain administered via the oral route (Figure 4-14). Bacterial penetration to the mLNs was significantly higher in Apc$^{Min/+}$ mice also when the non-invasive *Salmonella* strain was used. Moreover, ileal polyps contained higher bacterial loads than the ileal tissue from either Apc$^{Min/+}$ and littermate mice, while bacterial titres recovered from the spleen were also in this case identical (Figure 4-14). This indicates that the higher bacterial penetrance observed in Apc$^{Min/+}$

mice was due to leakiness of the intestinal barrier rather than to the invasiveness of the bacterial strain used. The intestinal barrier could in this frame display higher permeability also to components of the endogenous microbial flora upon tumorigenesis.

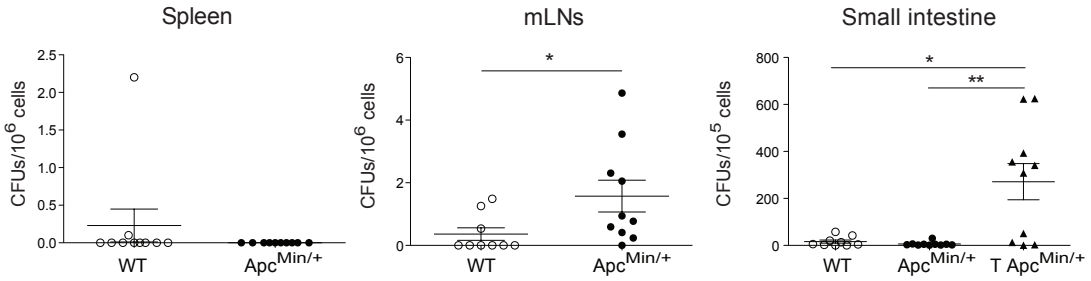

Figure 4-14 Permeability to bacterial challenge is altered in Apc$^{Min/+}$ mice also when a non-invasive *Salmonella* strain is used. 16 weeks old WT and Apc$^{Min/+}$ mice were orally infected with 10^9 CFUs of invA ΔaroA *S.* typhimurium. CFU load in the spleen, mLN and SI was addressed at 72 hpi. Graphs show mean ± SEM. n=9/10. * p<0,05, ** p<0,01.

Infection of WT and Apc$^{Min/+}$ mice *via* intravenous route with the ΔaroA *S.* typhimurium resulted in similar bacterial spreading profiles to the mLNs, implying that the differences we observe when using the oral administration route are due to an alteration of the intestinal barrier properties rather than intrinsic capacity to respond to the infection (Figure 4-15). However we observed massive bacterial localization in Apc$^{Min/+}$ polyps, confirming the tropism of this bacterium for tumoral lesions (Pawelek et al., 2003).

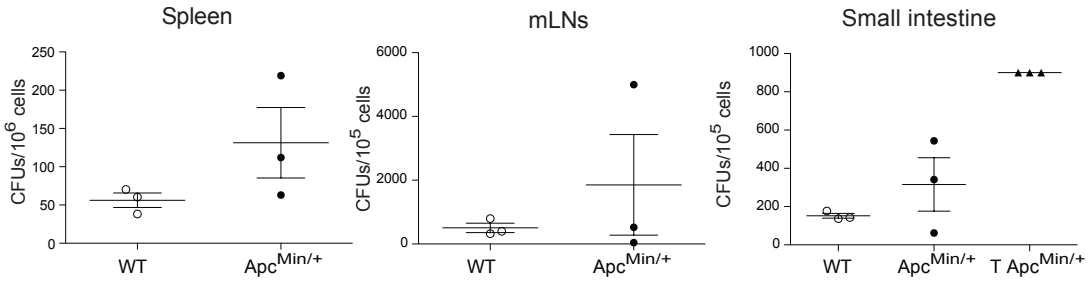

Figure 4-15 Infection via the intravenous route leads to similar bacterial spreading in WT and Apc$^{Min/+}$ mice. 16 weeks old WT and Apc$^{Min/+}$ mice were intravenously infected with $5x10^4$ CFUs of ΔaroA *S.* typhimurium. CFU load in the spleen, mLN and SI was addressed at 72 hpi. Graphs show mean ± SEM. n=4/6. * p<0,05, ** p<0,01.

4.3.2 Apc$^{Min/+}$ mice develop an IgA-skewed response upon oral vaccination with *Salmonella*

Having observed altered barrier properties in Apc$^{Min/+}$ mice we analysed their ability to mount a mucosal response after oral vaccination with ΔaroA *S.* typhimurium. WT and Apc$^{Min/+}$ mice were orally challenged every other day for three times with *Salmonella* and mice were followed-up for 6 weeks for the development of antibody titres in the serum and in the feaces (Figure 4-16, Figure 4-17). We found that Apc$^{Min/+}$ mice developed higher *Salmonella*-specific IgA titres in the serum when compared to WT mice while antigen specific IgG where lower (Figure 4-16). The total IgA titre was comparable between the two strains for the whole time course (Figure 4-17). At the fecal level there was no significant difference in the *Salmonella*-specific IgA response elicited by the vaccination (Figure 4-17).

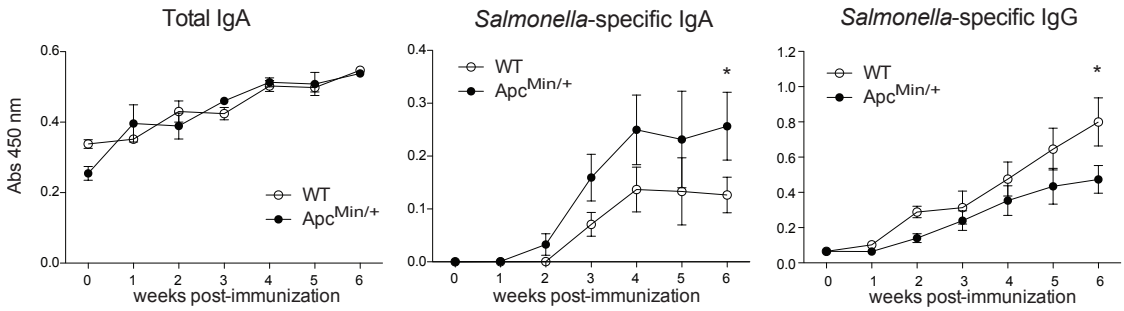

Figure 4-16 Apc$^{Min/+}$ mice develop higher *Salmonella*-specific IgA titres upon oral vaccination. 8 weeks old WT and Apc$^{Min/+}$ mice were vaccinated with 3 oral challenges of 10^9 CFUs of ΔaroA *S.* typhimurium every other day. Total IgA and *Salmonella*-specific IgA and IgG in the sera were evaluated by ELISA assay. Graphs show mean ± SEM. n=6. * p<0,05.

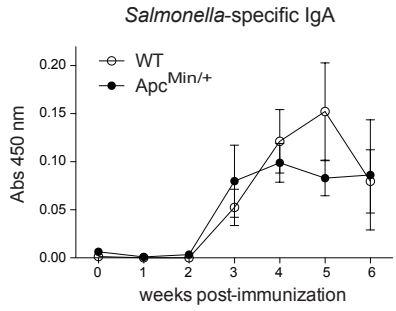

Figure 4-17 Fecal *Salmonella*-specific IgA titres are similar in WT and Apc$^{Min/+}$ mice. 8 weeks old WT and Apc$^{Min/+}$ mice were vaccinated with 3 oral challenges of 10^9 CFUs of ΔaroA *S.* typhimurium every other

day. *Salmonella*-specific IgA and IgG in the sera were evaluated by ELISA assay. Graph shows mean ± SEM. n=6.

6 weeks after the vaccination mice were challenged with ΔaroA *S.* typhimurium and 24 hours later serum was collected and the level of circulating alanine transaminase (ALT) evaluated (Figure 4-18). Vaccinated $Apc^{Min/+}$ mice displayed circulating ALT levels similar to untreated mice, while vaccinated WT mice had significantly increased ATL levels (Figure 4-18). The lower ALT level in vaccinated $Apc^{Min/+}$ mice is probably due to the protection to the rechallenge conferred by the higher *Salmonella*-specific IgA titres.

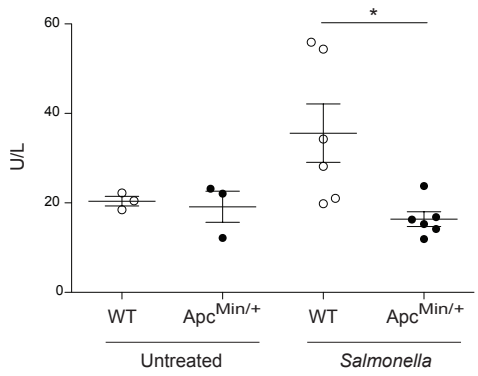

Figure 4-18 Vaccinated $Apc^{Min/+}$ mice are less susceptible to liver damage upon *Salmonella* rechallenge. 8 weeks old WT and $Apc^{Min/+}$ mice were vaccinated with 3 oral challenges of 10^9 CFUs of ΔaroA *S.* typhimurium every other day. After 6 weeks mice were rechallenged with 10^9 CFUs of ΔaroA *S.* typhimurium. 24h later seric ALT levels were measured with colorimetric assay. Graph shows mean ± SEM. n=6. * p<0,05.

Taken together these data show that there is higher bacterial penetrance at the intestinal level in $Apc^{Min/+}$ mice compared to WT littermates, leading to higher spreading to the mLNs. This higher permeability could lead to the development of higher IgA titres upon vaccination. *Salmonella*-specific IgA are protective towards rechallenge as shown by reduced ATL levels in vaccinated $Apc^{Min/+}$ mice upon rechallenge.

4.4 Disruption of the mucus barrier enhances intestinal tumorigenesis

It has been demonstrated that genetic ablation of the Muc2 gene leads to spontaneous colorectal carcinogenesis in mice (Velcich et al., 2002). Moreover ablation of Muc2 in murine models of Apc-driven intestinal tumorignesis increases the phenotype both in the small intestine and in the colon (Yang et al., 2008). We investigated whether exogenous disruptions of the mucus layer had an effect on tumorigenesis. We used two different approaches to disrupt the endogenous mucus barrier. The first was by orally administering *Salmonella*, a bacterium that binds to and degrades the mucus (McCormick et al., 1988; Nevola et al., 1987; Ouwerkerk et al., 2013; Vimal et al., 2000). In the second approach we employed N-acetyl cysteine treatment coupled to the chemically induced colitis associated CRC model AOM/DSS.

4.4.1 Repeated *Salmonella* challenges favour colon tumorigenesis in the $Apc^{Min/+}$ model

Salmonella is able to bind and degrade the mucus (McCormick et al., 1988; Nevola et al., 1987; Ouwerkerk et al., 2013; Vimal et al., 2000). To address whether *Salmonella*-induced mucus perturbation was altering intestinal tumorigenesis we orally administered ΔaroA *S.* typhimurium to littermate and $Apc^{Min/+}$ mice starting at 8 weeks of age once a week for a total of four administrations. *Salmonella* was administered either alone or after treating the mice with streptomycin, according to a published protocol of *Salmonella*-induced colitis (Barthel et al., 2003). Mice were followed up for further four weeks and sacrificed at 16 weeks of age.

Salmonella-specific antibody titres were evaluated in the serum and faeces over the course of the experiment (Figure 4-19). $Apc^{Min/+}$ mice treated with streptomycin and *Salmonella* developed higher seric antigen-specific IgA levels, while seric IgG were comparable among all the experimental groups (Figure 4-19). At the fecal level, both

WT and Apc$^{Min/+}$ mice treated with streptomycin and *Salmonella* had higher *Salmonella*-specific IgA titres compared with mice treated only with *Salmonella* (Figure 4-19).

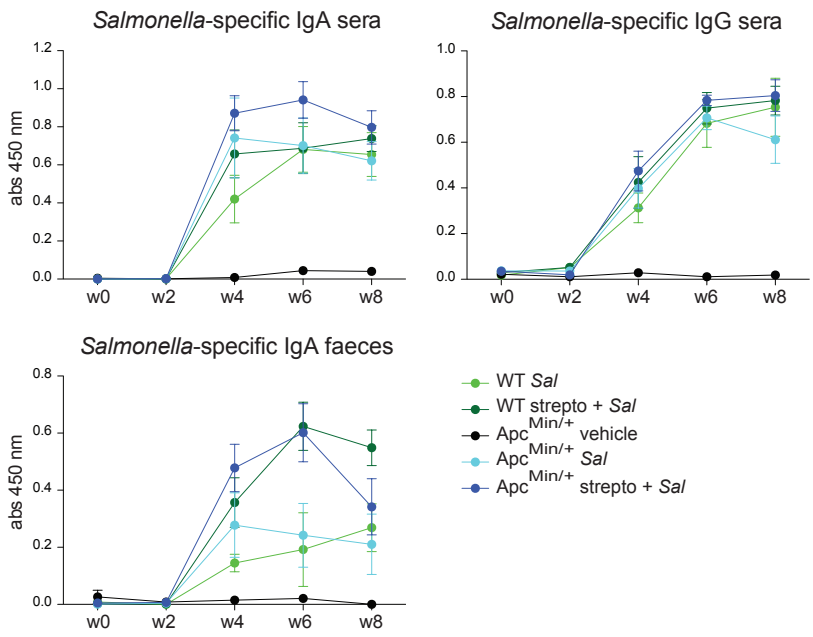

Figure 4-19 *Salmonella*-specific antibody titres in treated WT and Apc$^{Min/+}$ mice. 8 weeks old WT and Apc$^{Min/+}$ mice were orally challenged with vehicle or 10^9 CFUs of ΔaroA *S.* typhimurium either coupled or not with streptomycin pretreatment. The treatment was repeated for 4 times in 4 consecutive weeks and mice were followed-up for further 4 weeks. *Salmonella*-specific IgA and IgG were detected in sera and faeces by ELISA assay. Graphs show mean ± SEM. n=6/7.

Neither of the two infection protocol used had an effect on the polyp multiplicity in the SI of Apc$^{Min/+}$ mice (Figure 4-20). On the contrary tumorigenesis in the colon of mice treated with *Salmonella*, either alone or in combination with streptomycin, was increased (Figure 4-20).

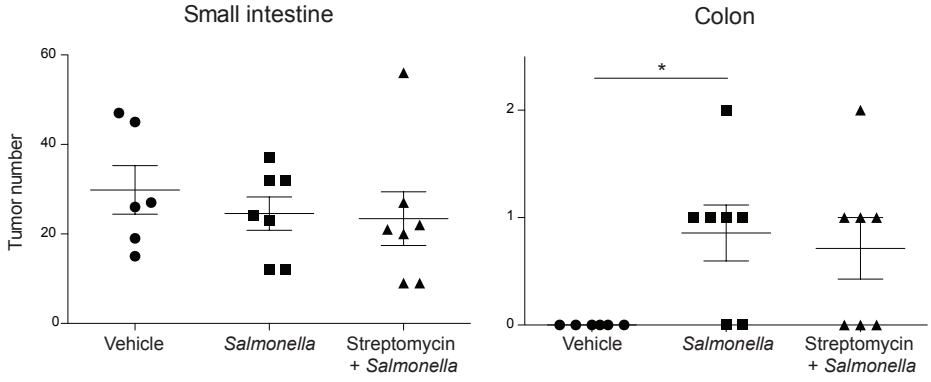

Figure 4-20 *Salmonella* administration enhances colon tumorigenesis in the colon of $Apc^{Min/+}$ mice. 8 weeks old WT and $Apc^{Min/+}$ mice were orally treated with carbonate buffer alone (Vehicle), 10^9 CFUs of ΔaroA *S.* typhimurium (*Salmonella*) or streptomycin and *Salmonella* (Streptomycin + *Salmonella*) once a week for four consecutive weeks. Mice were sacrificed at 16 weeks of age and tumor multiplicity in the small intestine and colon was assessed. Graphs show mean ± SEM. n=6/7. * p<0,05.

These data demonstrate that treatment with a mucus-degrading microorganism can enhance intestinal tumorigenesis. The specific effect of *Salmonella* in inducing colonic rather that small intestinal polyps is possibly due to its preferential localization in the caecum and colon rather than in the SI and to the fact that polyposis might be at saturation in the SI as observed by Newman and colleagues in *C. rodentium* treated $Apc^{Min/+}$ mice (Newman et al., 2001).

4.4.2 Disruption of the mucus layer is detrimental in AOM/DSS colon carcinogenesis model

The AOM/DSS model is widely used as model of chemically induced colitis associated colorectal carcinogenesis (Neufert et al., 2007). To address whether mucus disruption with a chemical agent had an effect on colonic tumorigenesis we modified the classical AOM/DSS protocol giving the mice a mucolytic agent (N-acetyl cysteine, NAC 1%) dissolved in the drinking water during the recovery phases. Mice treated with AOM/DSS and NAC had worsened disease symptoms during the course of the experiment, as documented by increased weight loss (Figure 4-21) and higher mortality (Figure 4-22).

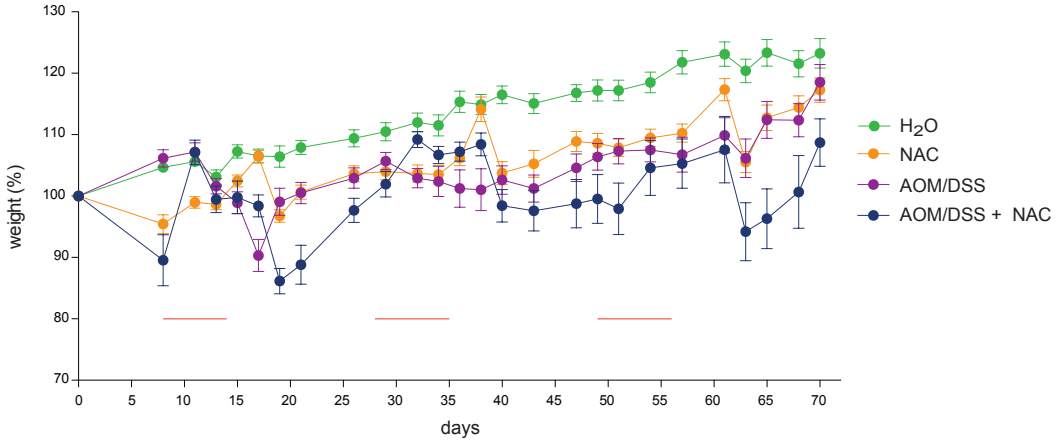

Figure 4-21 Weight curve of AOM/DSS mice treated or not with mucolytic agent. C57BL/6 females (n=10) aged 8 weeks were treated with azoxymethane (AOM). After 7 days control groups received respectively nothing and NAC 1% in drinking water (groups AOM and NAC). Two cohorts received dextran sodium sulphate (DSS) 2% in drinking water for 7 days (AOM/DSS and AOM/DSS NAC). Both groups were allowed for recovery for 14 days but while group AOM/DSS received water upon DSS stop, group AOM/DSS NAC received NAC 1%. This schedule was repeated for 3 cycles. Weight was monitored throughout the experiment and represented as % of the initial body weight. Graph shows mean ± SEM. n=10.

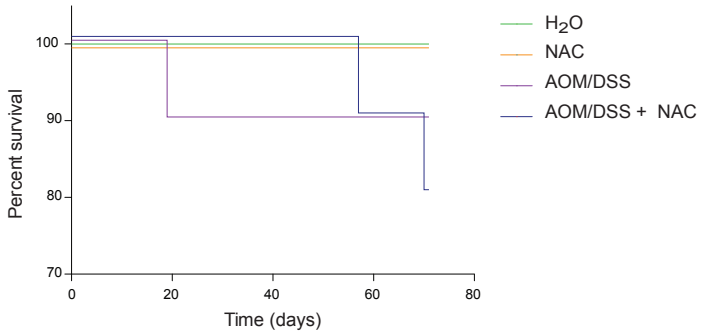

Figure 4-22 Weight curve of AOM/DSS mice treated or not with mucolytic agent. C57BL/6 females (n=10) aged 8 weeks were treated with azoxymethane (AOM). After 7 days control groups received respectively nothing and NAC 1% in drinking water (groups AOM and NAC). Two cohorts received dextran sodium sulphate (DSS) 2% in drinking water for 7 days (AOM/DSS and AOM/DSS NAC). Both groups were allowed for recovery for 14 days but while group AOM/DSS received water upon DSS stop, group AOM/DSS NAC received NAC 1%. This schedule was repeated for 3 cycles. Survival was monitored daily. n=10.

At the end of the experiment colons were analysed for tumor multiplicity and histological parameters. Tumor multiplicity was only slightly increased in the mucolytic treated group (Figure 4-23). Histological analysis revealed that colitis grade and the number of ulcerative lesions and dysplastic glands was comparable with or without mucolytic treatment, however more polypoid adenoma/carcinoma lesions were observed in the mucolytic treated group (Figure 4-24). These results confirm that disruption of the mucus layer is detrimental to colonic tumorigenesis. Importantly the comparable inflammation grade observed in the mucolytic treated group compared to the classical AOM/DSS protocol rules out possible anti-inflammatory effects due to NAC treatment.

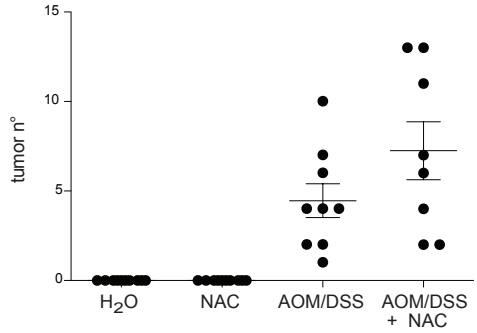

Figure 4-23 Tumor multiplicity is slightly increased in the mucolytic treated AOM/DSS group. C57BL/6 females (n=10) aged 8 weeks were treated with azoxymethane (AOM). After 7 days control groups received respectively nothing and NAC 1% in drinking water (groups AOM and NAC). Two cohorts received dextran sodium sulphate (DSS) 2% in drinking water for 7 days (AOM/DSS and AOM/DSS NAC). Both groups were allowed for recovery for 14 days but while group AOM/DSS received water upon DSS stop, group AOM/DSS NAC received NAC 1%. This schedule was repeated for 3 cycles. At the end of the experiment tumor multiplicity in the colon was assessed. Graphs show mean ± SEM. n=10.

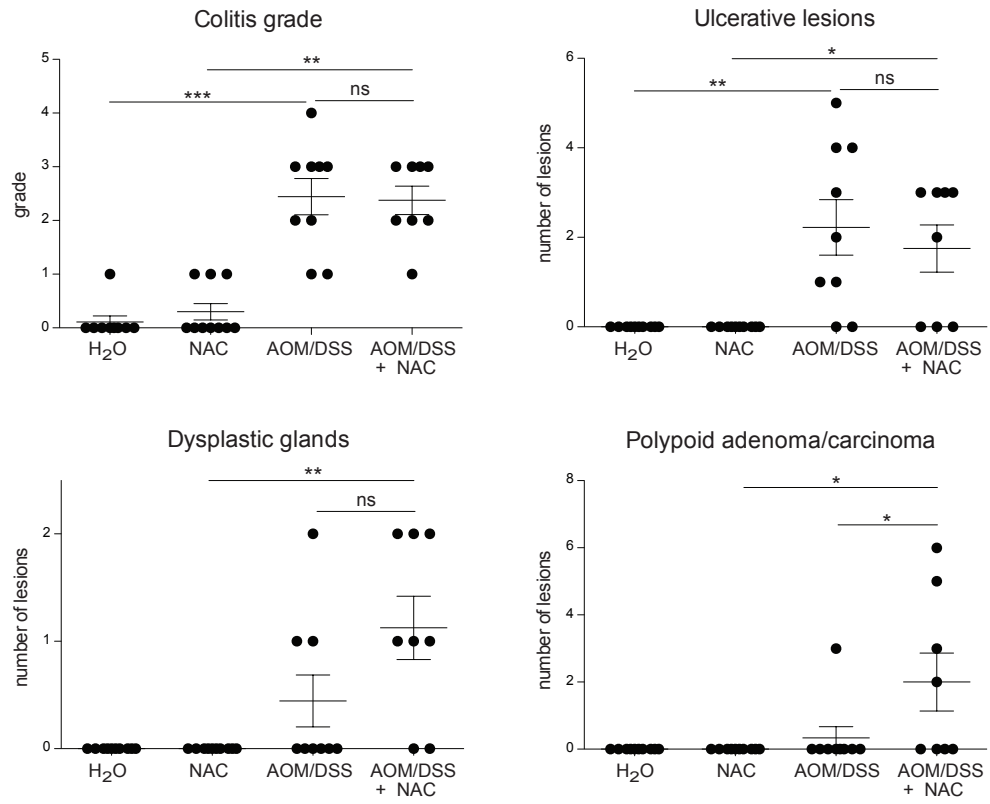

Figure 4-24 Mucolytic treatment increases the number of adenocarcinoma lesions in the AOM/DSS model. C57BL/6 females (n=10) aged 8 weeks were treated with azoxymethane (AOM). After 7 days control groups received respectively nothing and NAC 1% in drinking water (groups AOM and NAC). Two cohorts received dextran sodium sulphate (DSS) 2% in drinking water for 7 days (AOM/DSS and AOM/DSS NAC). Both groups were allowed for recovery for 14 days but while group AOM/DSS received water upon DSS stop, group AOM/DSS NAC received NAC 1%. This schedule was repeated for 3 cycles. At day 70 mice were sacrificed and histological parameters evaluated in the colon. Panels show colitis grade,

ulcerative lesions, dysplastic glands and polypoid adenoma/adenocarcinoma lesions as detected by histological evaluation. Graphs show mean ± SEM. n=10. * p<0,05; ** p<0,01; *** p<0,001.

4.4.3 Mucolytic treatment slightly alters mucin expression and does not modify mucus sulphonation status

Having observed that mucolytic treatment exacerbates colonic tumorigenesis in the AOM/DSS model we wondered what specific alteration in the mucus barrier were elicited by this treatment. To this end we treated C57BL/6 mice with plain water or NAC1% for four consecutive weeks and addressed mucin expression both in the small intestine and colon of these mice. RT-qPCR assay in the small intestine and colon revealed no major changes in the expression level of the mucins analysed (Figure 4-25, Figure 4-26). However, interestingly, we observed a slight reduction in Muc13 and increase in Muc20 expression (Figure 4-25) similarly to what we observe in the $Apc^{Min/+}$ ilei (Figure 4-6). In the colon we observed a tendency towards an increase in both Muc13 and Muc20 and a slight reduction in Muc3 expression level (Figure 4-26).

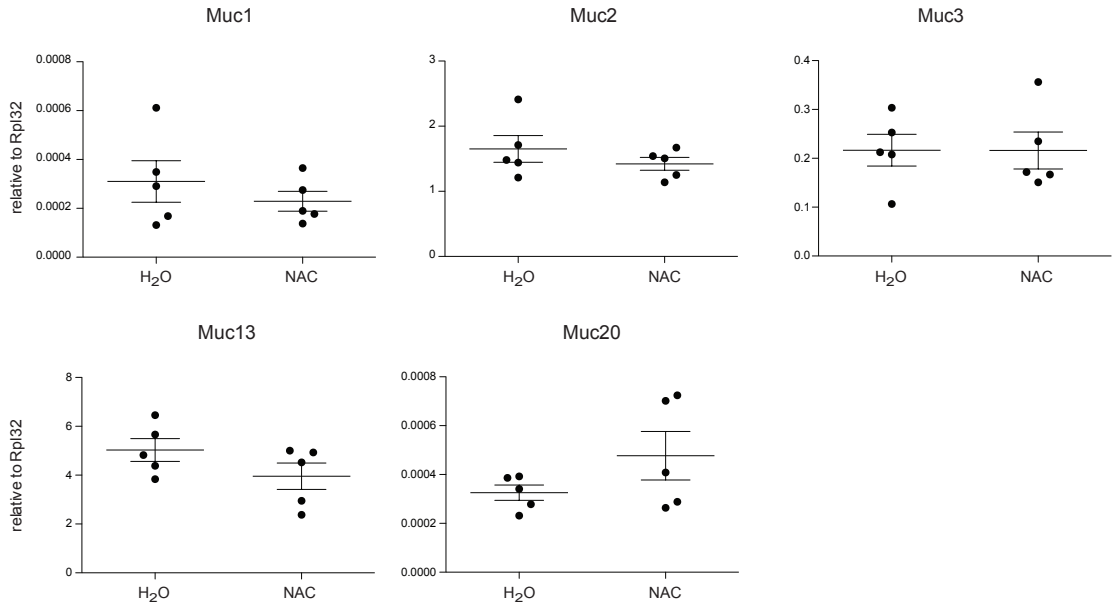

Figure 4-25 Mucolytic treatment slightly modifies mucin expression in the ileum. C57BL/6 mice were treated either with plain water (H_2O) or with NAC 1% (NAC) in drinking water for four weeks. Expression levels for Muc1, Muc2, Muc3, Muc13 and Muc20 were evaluated by RT-qPCR assay on ileal tissue. Expression levels are normalized to Rpl32. n=5. Graphs show mean ± SEM.

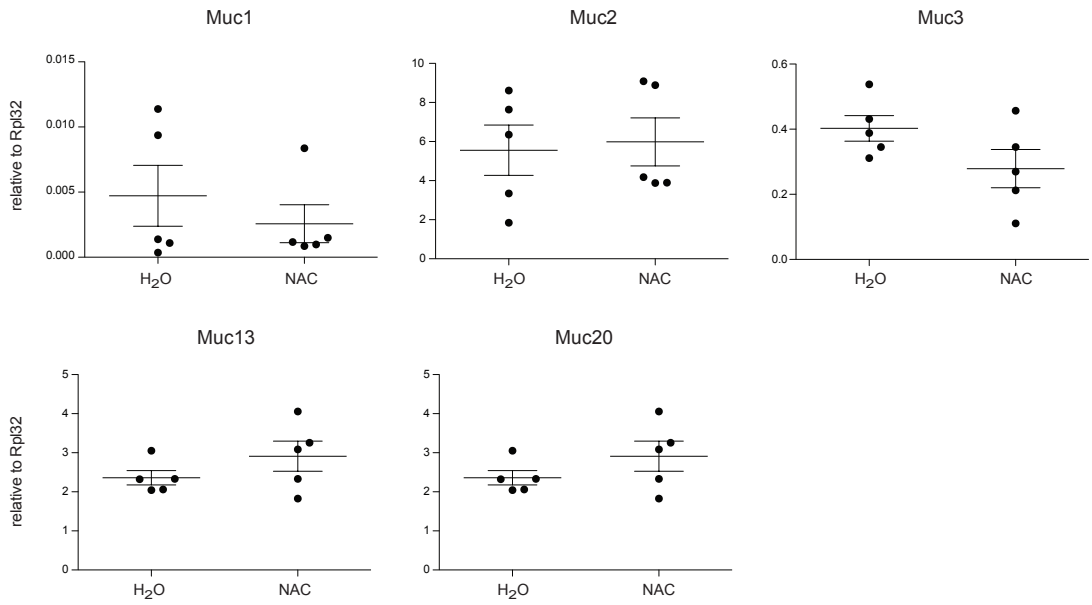

Figure 4-26 Mucolytic treatment slightly modifies mucin expression in the colon. C57BL/6 mice were treated either with plain water (H_2O) or with NAC 1% (NAC) in drinking water for four weeks. Expression levels for Muc1, Muc2, Muc3, Muc13 and Muc20 were evaluated by RT-qPCR assay on colon tissue. Expression levels are normalized to Rpl32. n=5. Graphs show mean ± SEM.

We next addressed whether mucus sulphonation status could be altered by mucolityc treatment. AB/HID staining on ileal and colonic sections of mice treated or not with NAC 1% showed no major differences in sulphonation level (Figure 4-27, Figure 4-28). However the AB staining clearly showed that NAC 1% treatment is effective in reducing the intestinal mucus layer (Figure 4-27, Figure 4-28).

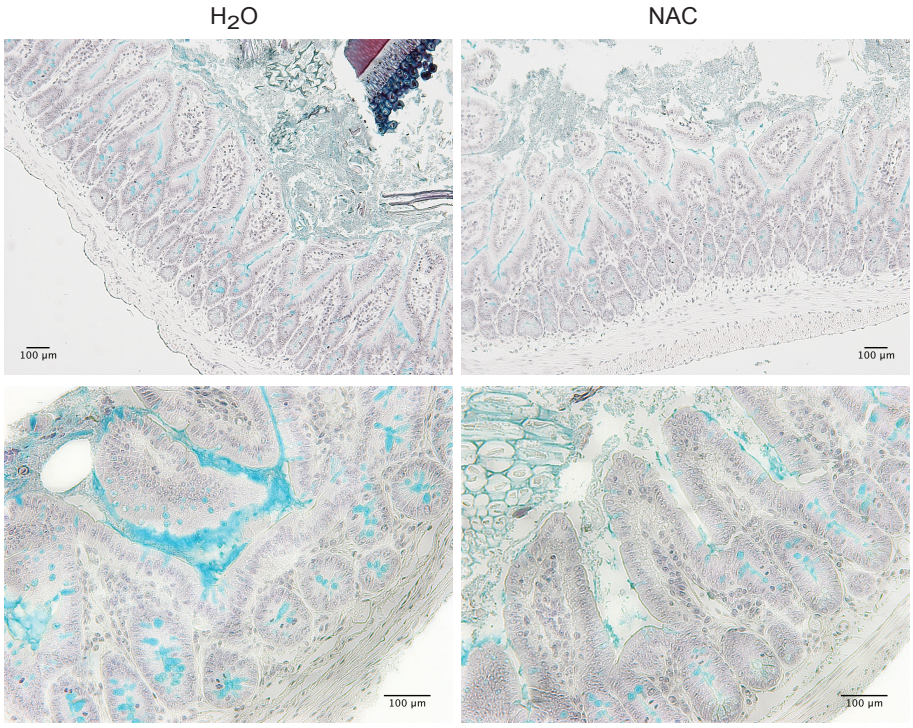

Figure 4-27 Mucolytic treatment does not alter sulphonation level in the small intestine. C57BL/6 mice were treated either with plain water (H_2O) or with NAC 1% (NAC) in drinking water for four weeks. 4 µm ileal sections were stained with AB/HID. Blue: sialomucins, black: sulphomucins. Representative pictures are shown.

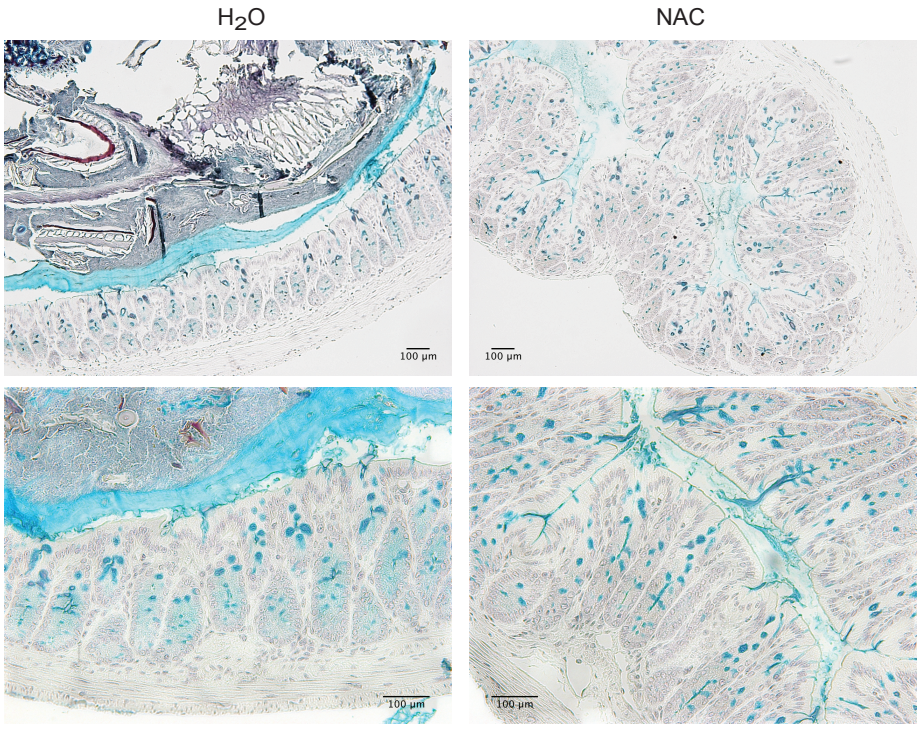

Figure 4-28 Mucolytic treatment does not alter sulphonation level in the colon. C57BL/6 mice were treated either with plain water (H_2O) or with NAC 1% (NAC) in drinking water for four weeks. 4 µm colon

sections were stained with AB/HID. Blue: sialomucins, black: sulphomucins. Representative pictures are shown.

4.5 Dysbiosis is an early event associated with intestinal tumorigenesis

4.5.1 Metagenomic analysis reveals dysbiosis in young $Apc^{Min/+}$ mice

Increasing evidence is mounting of the involvement of the microbiota in intestinal tumorigenesis (Zhu et al., 2013) and sequencing analysis on hCRC patients shows alterations of the microbial community associated with the tumor (Kostic et al., 2013). However a clear view of the microbial alterations occurring during tumorigenesis has not yet emerged, probably due to the heterogeneity of the samples analysed. Moreover studies on hCRC samples give a snapshot of the microbial community once the tumor has established, but they do not allow the identification and characterization of the moment of dysbiosis onset nor the dissection of the functional meaning of this for the tumorigenic process. As a matter of fact dysbiosis might only be a consequence of tumor formation and studies in the human system allow limited insight in the cause-effect relationships contributing to disease onset.

To get an insight in the microbiota dynamics during tumorigenesis we performed metagenomic analysis on $Apc^{Min/+}$ mice in a time course experiment. We collected fecal material from WT littermates and $Apc^{Min/+}$ mice at 4, 8, 12 and 16 weeks of age and sequenced the V5-V6 hypervariable regions of bacterial 16S rDNA. $Apc^{Min/+}$ mice were separated from WT littermates at 6 weeks of age to avoid microbiota exchange due to cohousing.

Richness and diversity indexes were calculated on both families and genera using different algorithms (Figure 4-29, Figure 4-30). The Chao1 index is a richness estimator. Our data showed that richness is comparable between WT and $Apc^{Min/+}$ mice both at family and genera level at all time points considered (Figure 4-29, Figure 4-30).

Shannon and Simpson indexes are diversity indexes that consider both richness and evenness within the population analysed. Shannon and Simpson diversity indexes calculated on both families and genera did not highlight differences between WT and $Apc^{Min/+}$ mice (Figure 4-29, Figure 4-30). This underlines that the $Apc^{Min/+}$ microbiome is comparable to the WT one in terms of richness and diversity over the time frame characterized by the onset and establishment of the tumorigenic process.

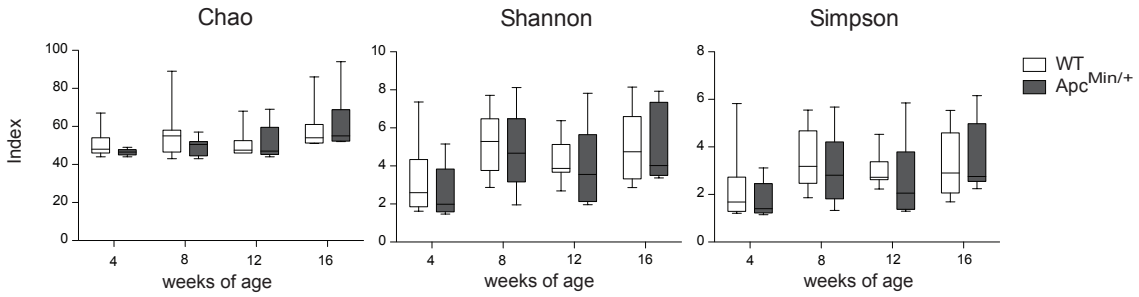

Figure 4-29 Diversity indexes relative to families. Metagenomic sequencing was performed on fecal samples from WT and $Apc^{Min/+}$ mice at 4, 8, 12 and 16 weeks of age. Chao, Shannon and Simpson diversity indexes relative to families are shown. n=8. Graphs show 5-95 percentile.

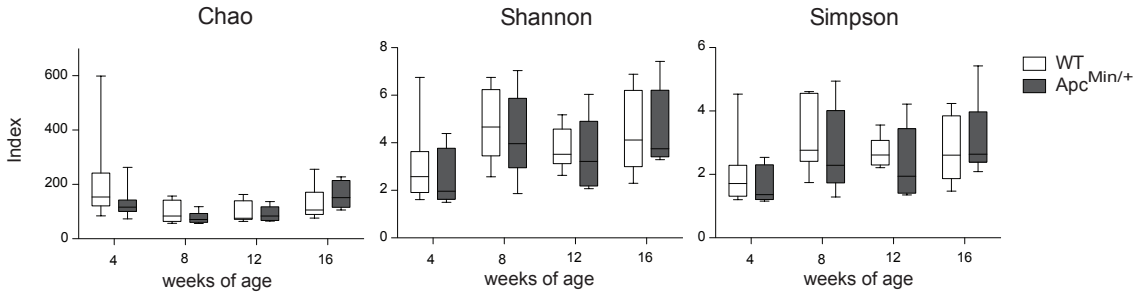

Figure 4-30 Diversity indexes relative to genera. Metagenomic sequencing was performed on fecal samples from WT and $Apc^{Min/+}$ mice at 4, 8, 12 and 16 weeks of age. Chao, Shannon and Simpson diversity indexes relative to genera are shown. n=8. Graphs show 5-95 percentile.

We then analysed genus abundance variations over the different time points tested (Figure 4-31). The results showed that $Apc^{Min/+}$ mice are characterized by a dysbiosis arising at very early time points. In fact $Apc^{Min/+}$ mice have an increase in *Lactobacillus* and a contraction in *Clostridium* already at 4 weeks of age (Figure 4-31). The same pattern is maintained at 8 and 12 weeks of age (Figure 4-31). At 16 weeks of age this

trend is inverted and *Lactobacillus* genus becomes less represented and *Clostridium* genus more represented in Apc$^{Min/+}$ mice compared to WT (Figure 4-31). This could be explained by the appearance at this late time point of symptoms related to tumorigenesis such as intestinal bleeding that could alter the luminal microenvironment and as a consequence the microbial community.

Appendix1 lists the principal genera identified by metagenomic sequencing and their statistical variation at the different time points analysed.

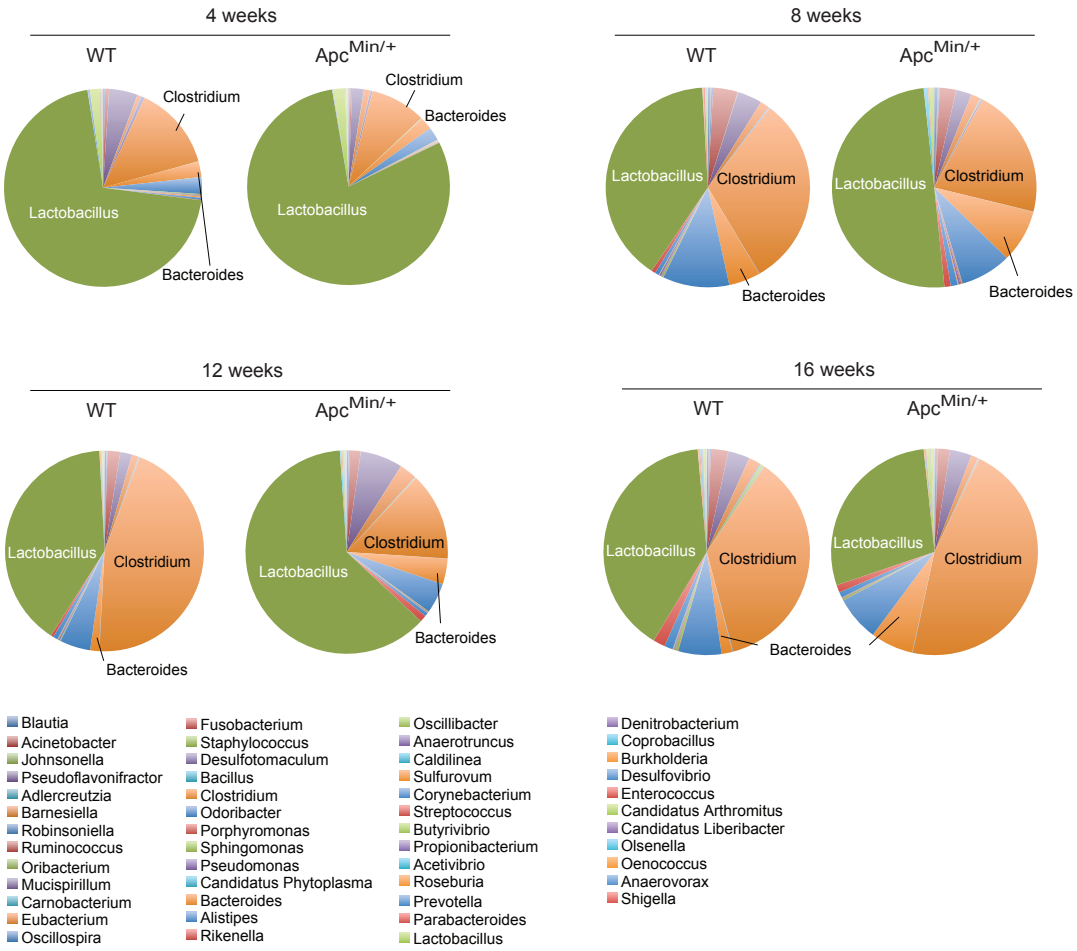

Figure 4-31 Genus abundance in WT and Apc$^{Min/+}$ mice. Metagenomic sequencing was performed on fecal samples from WT and Apc$^{Min/+}$ mice at 4, 8, 12 and 16 weeks of age. Genus abundance at the different time points is shown as pie chart. n=8.

4.5.2 *Clostridium* ID4 is underrepresented in Apc$^{Min/+}$ fecal and mucus-associated microbiome

We then compared metagenomic data of WT and Apc$^{Min/+}$ mice over the different time points to identify differences at the OTU level. We found that the species *Clostridium* ID4 is underrepresented in Apc$^{Min/+}$ samples at 8 and 12 weeks of age (Figure 4-32). *Clostridium* ID4 abundance was statistically reduced in Apc$^{Min/+}$ fecal samples when considering the normalised number of assigned sequences from the metagenomic analysis (Figure 4-32 A). These data were validated with qPCR assay using *C*. ID4 specific primers (Figure 4-32 B). As observed before for the *Clostridium* genus (Figure 4-31) also *C*. ID4 abundance dramatically increased at 16 weeks of age (Figure 4-32).

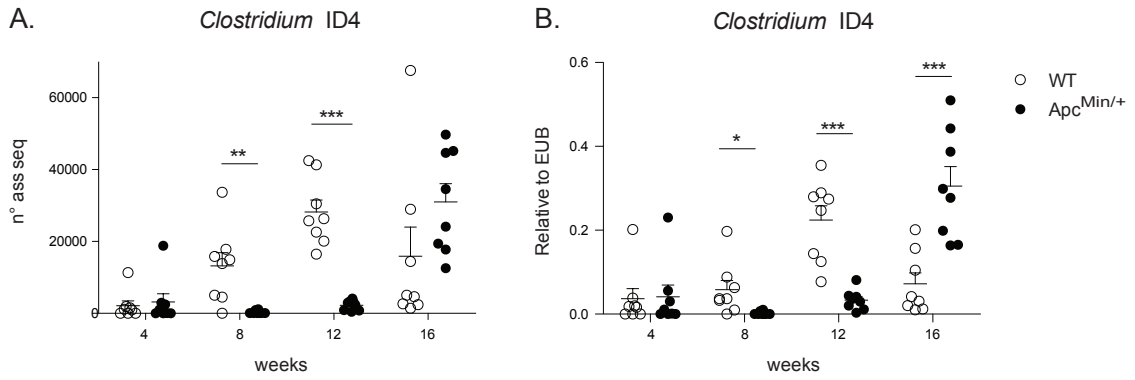

Figure 4-32 *Clostridium* ID4 abundance in WT and Apc$^{Min/+}$ mice. DNA was extracted from faeces of WT and Apc$^{Min/+}$ mice at 4, 8 and 12 weeks of age. Graphs show the **A**. normalised number of assigned sequences in the metagenomic analysis and **B**. as detected by qPCR valiation assay (left panel). *C*. ID4 abundance levels detected by qPCR levels are normalized to Eubacteria (EUB). Graphs show mean ± SEM. n=8. * p<0,05. **p<0,01. ***p<0,001.

Since the mucus constitutes a homing niche to many intestinal microorganisms (Derrien et al., 2010; Johansson et al., 2008; Ouwerkerk et al., 2013) we addressed whether the variation of *C*. ID4 observed at fecal level was present also in the mucus-associated community (Figure 4-33). DNA was extracted from faeces and scraped mucus from both the small intestine and colon of WT and Apc$^{Min/+}$ mice at 4, 8 and 12 weeks of age. *C*. ID4 abundance was evaluated by qPCR assay. Interestingly we found that the depletion of *C*. ID4 that we observed at 8 and 12 weeks of age at the fecal level parallels with a

reduction also at the level of mucus-associated bacteria (Figure 4-33). These results show that as far as *Clostridium* ID4 abundance is concerned we can consider the dynamics observed in the faeces representative of the mucus-associated community.

In this experiment *C*. ID4 level was slightly but not significantly reduced in fecal and mucus samples from $Apc^{Min/+}$ mice at 8 weeks of age (Figure 4-33), while in the previous experiment it was significantly depleted by that time point (Figure 4-32). This could be explained by the fact that in this second experiment $Apc^{Min/+}$ mice were separated from WT littermates at a later time point, namely 7 weeks of age, allowing microbiota exchange for a longer time.

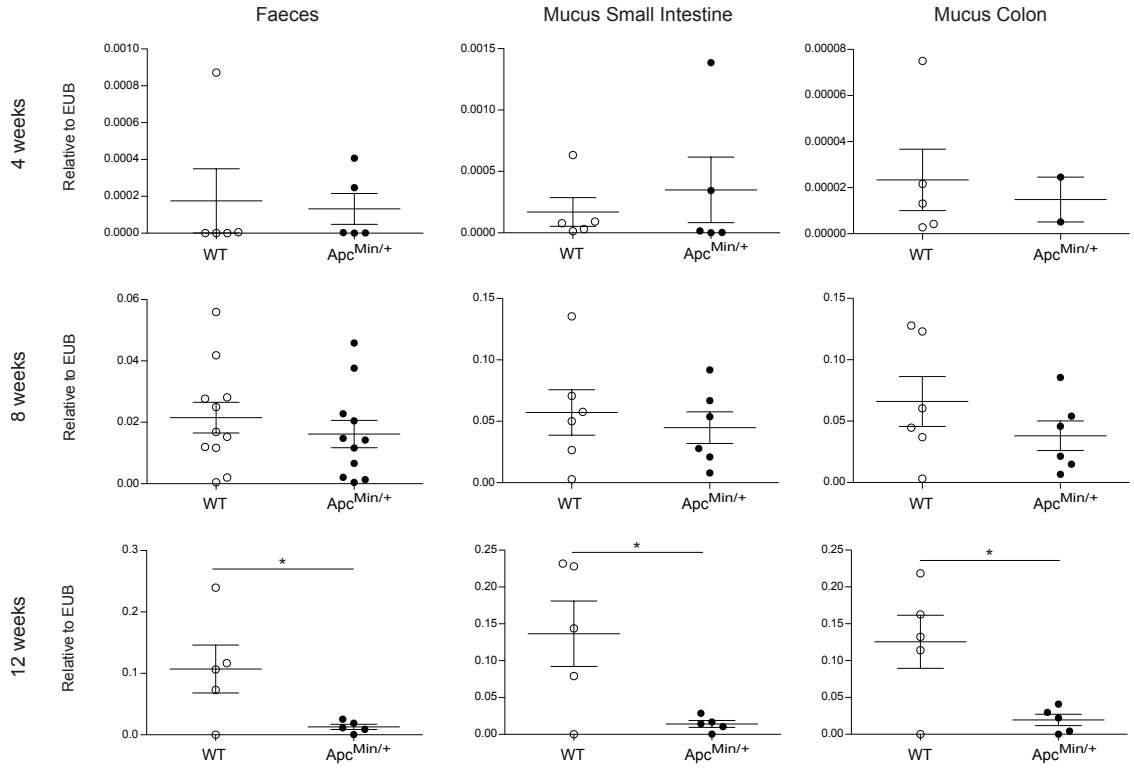

Figure 4-33 *Clostridium* ID4 abundance is altered also in the mucus-associated community. DNA was extracted from faeces and mucus scraped from the small intestine and colon of WT and $Apc^{Min/+}$ mice at 4, 8 and 12 weeks of age. *C*. ID4 abundance was assessed by qPCR. *C*. ID4 abundance levels are normalized to Eubacteria (EUB). Graphs show mean ± SEM. n=5/11. * p<0,05.

4.6 *Clostridium* ID4 effects on the intestinal immune system and on intestinal tumorigenesis

There is mounting evidence regarding the involvement of specific components of the endogenous microbiota in the differentiation of immune cell subsets in the intestinal microenvironment (Atarashi et al., 2013; Atarashi et al., 2011a; Ivanov et al., 2009; Ivanov et al., 2008). In particular *Clostridium* species belonging to Clostridial groups IV, XIVa and XVIII have been functionally linked to the development of IL10 producing FoxP3 Treg cells in the colonic lamina propria (Atarashi et al., 2013; Atarashi et al., 2011a). Having observed that $Apc^{Min/+}$ mice display depletion in one *Clostridium* species, namely *Clostridium* ID4, we addressed the effect of *C*. ID4 on the intestinal immune system development with monocolonization experiments in germ free mice and on the tumorigenic process with exogenous administration experiments in the $Apc^{Min/+}$ model.

4.6.1 *Clostridium* ID4 effects on intestinal immune system development

To address the effect of *Clostridium* ID4 on the intestinal immune system development germ free ICR mice were monocolonized with *C*. ID4. Five weeks after gnotobiotic transfer the abundance of Tregs, IL17 and IFNγ producing cells in the small intestinal and colonic lamina propria was evaluated by flow cytometry (Figure 4-34, Figure 4-35, Figure 4-36). Germ free and SPF mice were analysed as controls. *C*. ID4 colonization did not alter the abundance of Treg cells in the SI lamina propria compared to germ-free mice (Figure 4-34). In the colon on the contrary mice monocolonized with *C*. ID4 had increased Tregs compared to germ free mice, even if these were still significantly lower than SPF mice (Figure 4-34). As far as Th17 cells are concerned *C*. ID4 significantly increased the SI lamina propria Th17 population, even if the levels are lower compared to SPF mice, while having no effect on colonic Th17 or on IL17 producing cells in the CD8 subset (Figure 4-35). No major differences were observed in the IFNγ producing CD4 and CD8 populations in *C*. ID4 monocolonized mice (Figure 4-36). *Clostridium* ID4

monocolonization shows thus a partial effect in the expansion of colonic Treg cells and small intestinal Th17 cells.

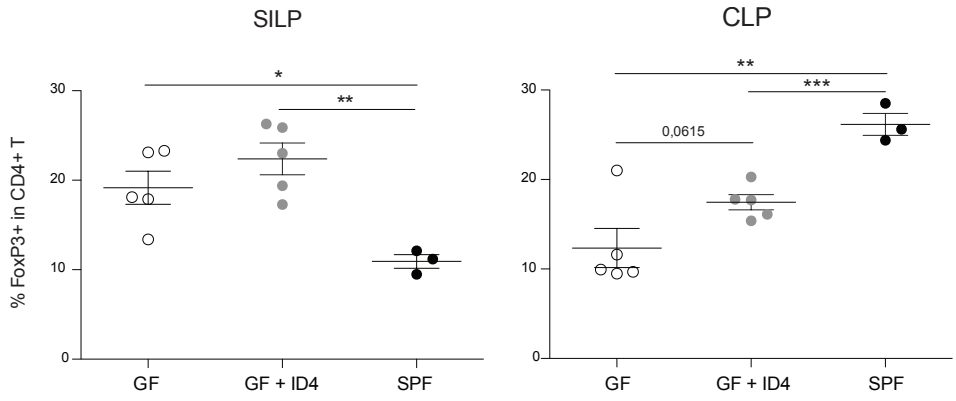

Figure 4-34 *Clostridium* ID4 administration slightly increases colonic Treg population. Germ free ICR mice were administered with *C.* ID4. 5 weeks later the small intestinal (SILP) and colonic lamina propria (CLP) of germ free (GF), *C.* ID4 monocolonized (GF + ID4) and SPF mice was analysed by flow cytometry for the presence of CD4+ FoxP3+ cells. Graphs show mean ± SEM. n=3/5. * p<0,05. ** p<0,01. *** p<0,001.

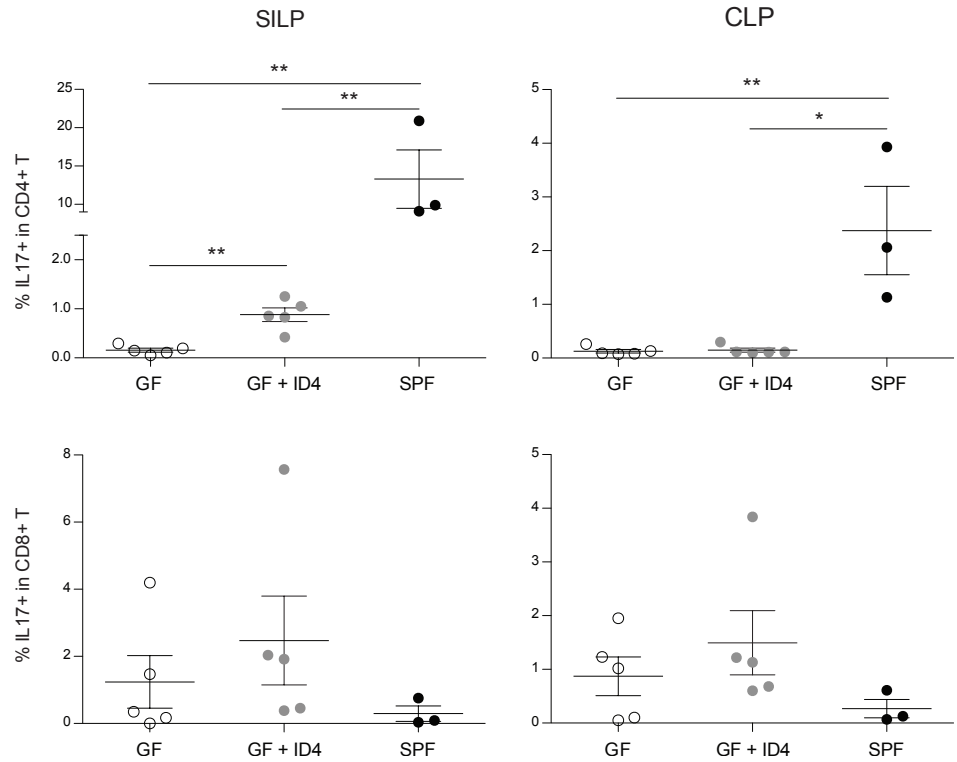

Figure 4-35 *Clostridium* ID4 administration increases small intestinal Th17 population. Germ free ICR mice were administered with *C.* ID4. 5 weeks later the small intestinal (SILP) and colonic lamina propria (CLP) of germ free (GF), *C.* ID4 monocolonized (GF + ID4) and SPF mice was analysed by flow cytometry

for the presence of IL17+ cells in the CD4 and CD8 subsets. Graphs show mean ± SEM. n=3/5. * p<0,05. ** p<0,01.

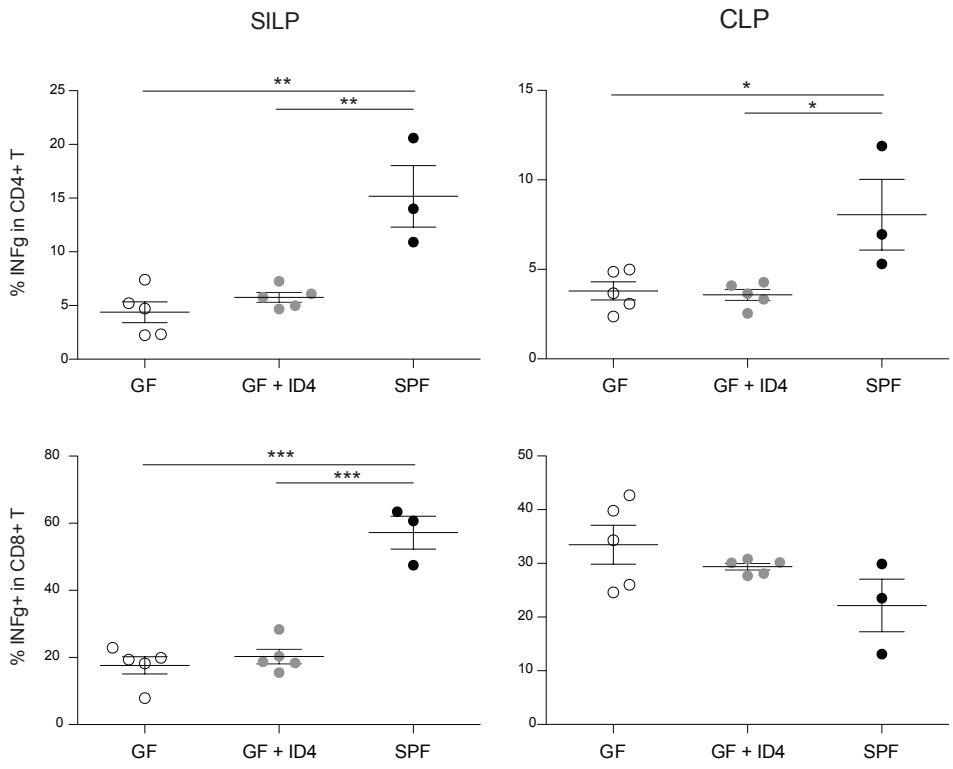

Figure 4-36 *Clostridium* ID4 administration has no effect on INFγ producing cells. Germ free ICR mice were administered with *C*. ID4. 5 weeks later the small intestinal (SILP) and colonic lamina propria (CLP) of germ free (GF), *C*. ID4 monocolonized (GF + ID4) and SPF mice was analysed by flow cytometry for the presence of INFγ+ cells in the CD4 and CD8 subsets. Graphs show mean ± SEM. n=3/5. * p<0,05. ** p<0,01. *** p<0,001

4.6.2 *Clostridium* ID4 effects on intestinal tumorigenesis

Metagenomic data and qPCR experiments showed that *Clostridium* ID4 is depleted in $Apc^{Min/+}$ mice in the time frame characterized by the onset of tumorigenesis (Figure 4-32 Figure 4-33). Having observed that monocolonization with *C*. ID4 partially increased the small intestinal Th17 population (Figure 4-35) and the colonic Treg population (Figure 4-34) and given the pro-tumorigenic role of the first population (Chae et al., 2010; Grivennikov et al., 2012; Song et al., 2014) and the controversial role of the second (Blatner et al., 2012; Gounaris et al., 2009; Keerthivasan et al., 2014) in the context of tumorigenesis we addressed the effect of exogenous re-colonization of $Apc^{Min/+}$ mice

with *C*. ID4. To this aim WT and Apc$^{Min/+}$ mice were administered twice a week with *C*. ID4 from 6 to 12 weeks of age. *C*. ID4 abundance in the faeces was assessed over the course of the experiment demonstrating that exogenous administration increased *C*. ID4 levels in the faeces of Apc$^{Min/+}$ mice even if these levels were lower than those observed in WT mice (Figure 4-37). Of note, *C*. ID4 levels in treated Apc$^{Min/+}$ mice were not significantly higher than in vehicle treated Apc$^{Min/+}$ mice (Figure 4-37), meaning that probably the bacterium does not find its niche in the Apc$^{Min/+}$ intestinal microenvironment. At 12 weeks of age mice were sacrificed and tumor multiplicity in the small intestine and colon assessed (Figure 4-38). *C*. ID4 treatment did only slightly alter the polyp number in the small intestine while having no effect in the colon (Figure 4-38).

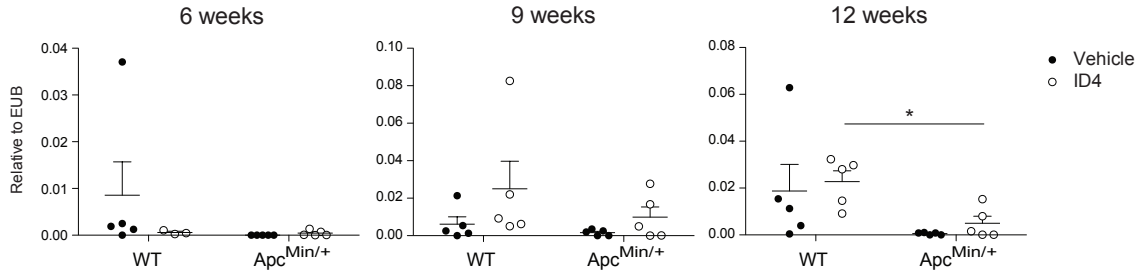

Figure 4-37 *Clostridium* ID4 abundance in the faeces of WT and Apc$^{Min/+}$ mice treated with vehicle or *C*. ID4. WT and Apc$^{Min/+}$ mice received either vehicle or *C*. ID4 (ID4) from 6 to 12 weeks of age. *C*. ID4 abundance in the faeces was assessed by qPCR. *C*. ID4 abundance levels are normalized to Eubacteria (EUB). Graphs show mean ± SEM. n=5. * p<0,05.

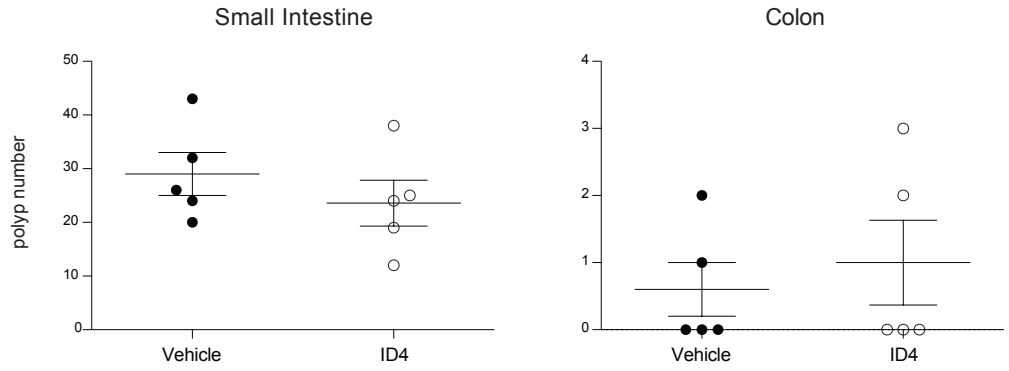

Figure 4-38 Tumor multiplicity in *Clostridium* ID4 treated Apc$^{Min/+}$ mice. Apc$^{Min/+}$ mice received either vehicle or *C*. ID4 (ID4) from 6 to 12 weeks of age. At 12 weeks of age mice were sacrificed and tumor multiplicity in the small intestine and colon assessed. Graphs show mean ± SEM. n=5.

Unpublished data from our laboratory show that high levels of neutrophils correlate with tumor number in the $Apc^{Min/+}$ model (Guglietta S., unpublished). We therefore analysed the abundance of inflammatory monocytes and neutrophils in WT and $Apc^{Min/+}$ mice treated either with vehicle or *C*. ID4 at the 12th week of age (Figure 4-39). Both inflammatory monocytes ($Ly6C^{high}$) and circulating neutrophils (Ly6G+ CD11b+) levels were not altered in *C*. ID4 treated $Apc^{Min/+}$ mice compared to vehicle treated ones (Figure 4-39). However *C*. ID4 administration reduced the level of circulating $Ly6C^{high}$ and in part neutrophils in WT mice (Figure 4-39).

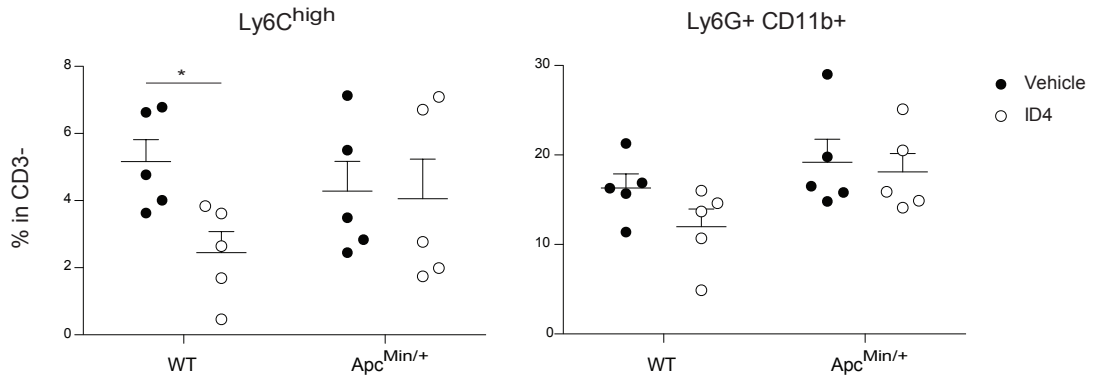

Figure 4-39 Inflammatory monocytes and neutrophil abundance in WT and $Apc^{Min/+}$ mice treated with vehicle or *C*. ID4. WT and $Apc^{Min/+}$ mice received either vehicle or *C*. ID4 (ID4) from 6 to 12 weeks of age. At 12 weeks of age the percentage of inflammatory monocytes (left panel: $Ly6G^{high}$ cells in the CD45+ CD3- gate) and neutrophils (right panel: Ly6G+ CD11b+ cells in the CD45+ CD3- gate) was assessed by flow cytometry. Graphs show mean ± SEM. n=5. * p<0,05.

Since Clostridia have been related to the differentiation of specific immune cell populations in the intestine (Atarashi et al., 2013; Atarashi et al., 2011a) we analysed the abundance of Tregs and IL17 or INFγ producing T cells in the small intestinal and colonic lamina propria of WT and $Apc^{Min/+}$ mice treated or not with *Clostridium* ID4 (Figure 4-40, Figure 4-41, Figure 4-42).

As far as the Treg population is concerned we observed that overall $Apc^{Min/+}$ mice have slightly higher nTregs (Helios+) in the small intestinal lamina propria compared to WT mice, independently of the treatment (Figure 4-40). *C*. ID4 treatment induced little expansion of the CD25+ FoxP3+ population in the small intestine of $Apc^{Min/+}$ mice (Figure

4-40). No major alteration was observed in the Treg population in the colon (Figure 4-40).

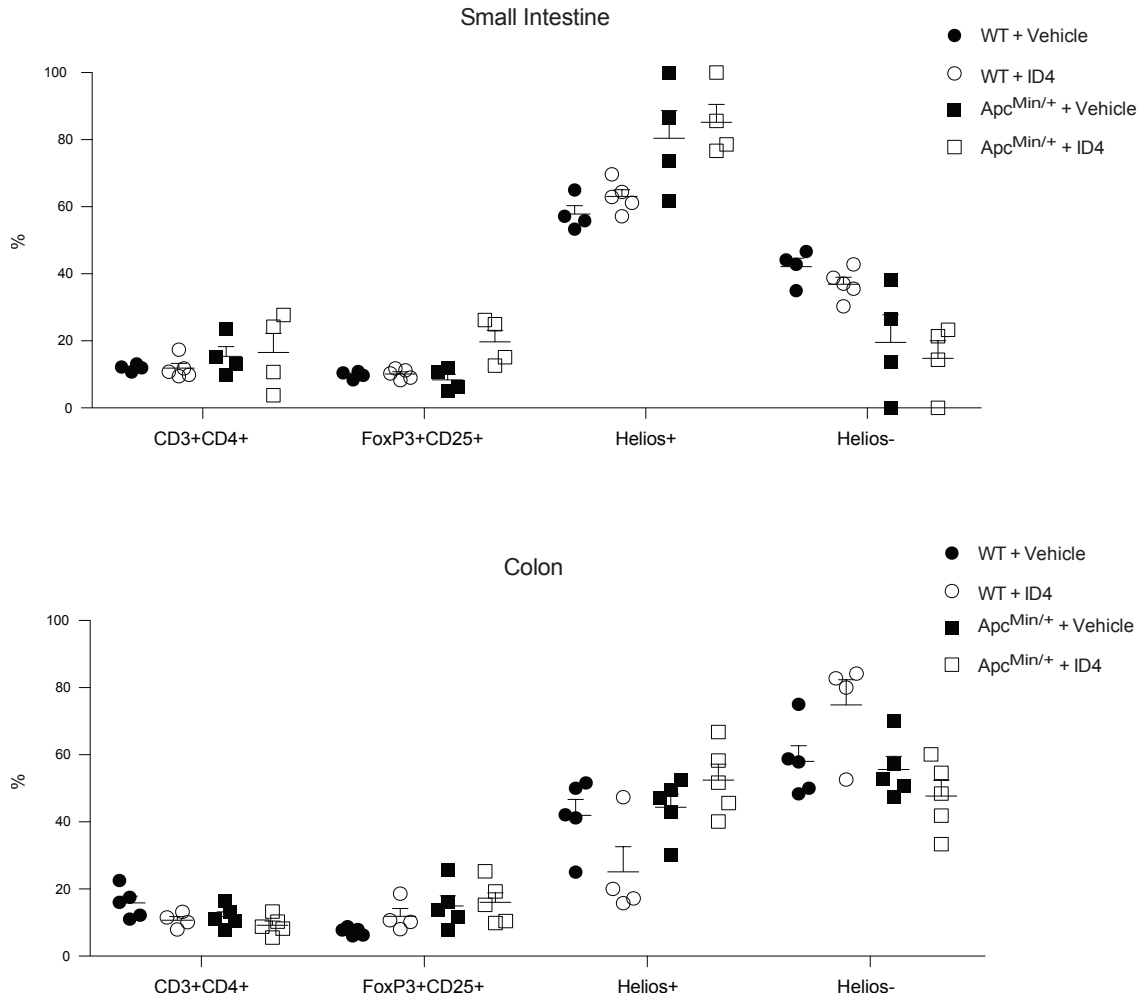

Figure 4-40 T regulatory cells in the small intestine and colon of WT and Apc$^{Min/+}$ mice treated with vehicle or *Clostridium* ID4. WT and Apc$^{Min/+}$ mice received either vehicle or *C*. ID4 (ID4) from 6 to 12 weeks of age. At 12 weeks of age cells were isolated from the small intestinal and colonic lamina propria and the percentage of FoxP3+ CD25+ cells (within the CD3+ CD4+ population) and Helios+ and Helios- cells (within the FoxP3+ CD25+ population) evaluated by flow cytometry. Graphs show mean ± SEM. n=5.

Analysis of the IL17 and IFNγ producing cells within the CD3+ CD4+ T helper subset did not point out differences elicited by *C*. ID4 treatment (Figure 4-41). However, irrespective of the treatment we observed higher IL17+ T helper cells in the small intestine of Apc$^{Min/+}$ mice compared to WT mice (p=0,026) (Figure 4-41). We did not observe differences in the CD8 compartment between vehicle and *C*. ID4 treated mice, nor between WT and Apc$^{Min/+}$ mice (Figure 4-42).

Clostridium ID4 exogenous administration is in conclusion producing no major effect on the abundance of Tregs and IL17 and IFNγ producing cells in the small intestine and colon of both WT and $Apc^{Min/+}$ mice.

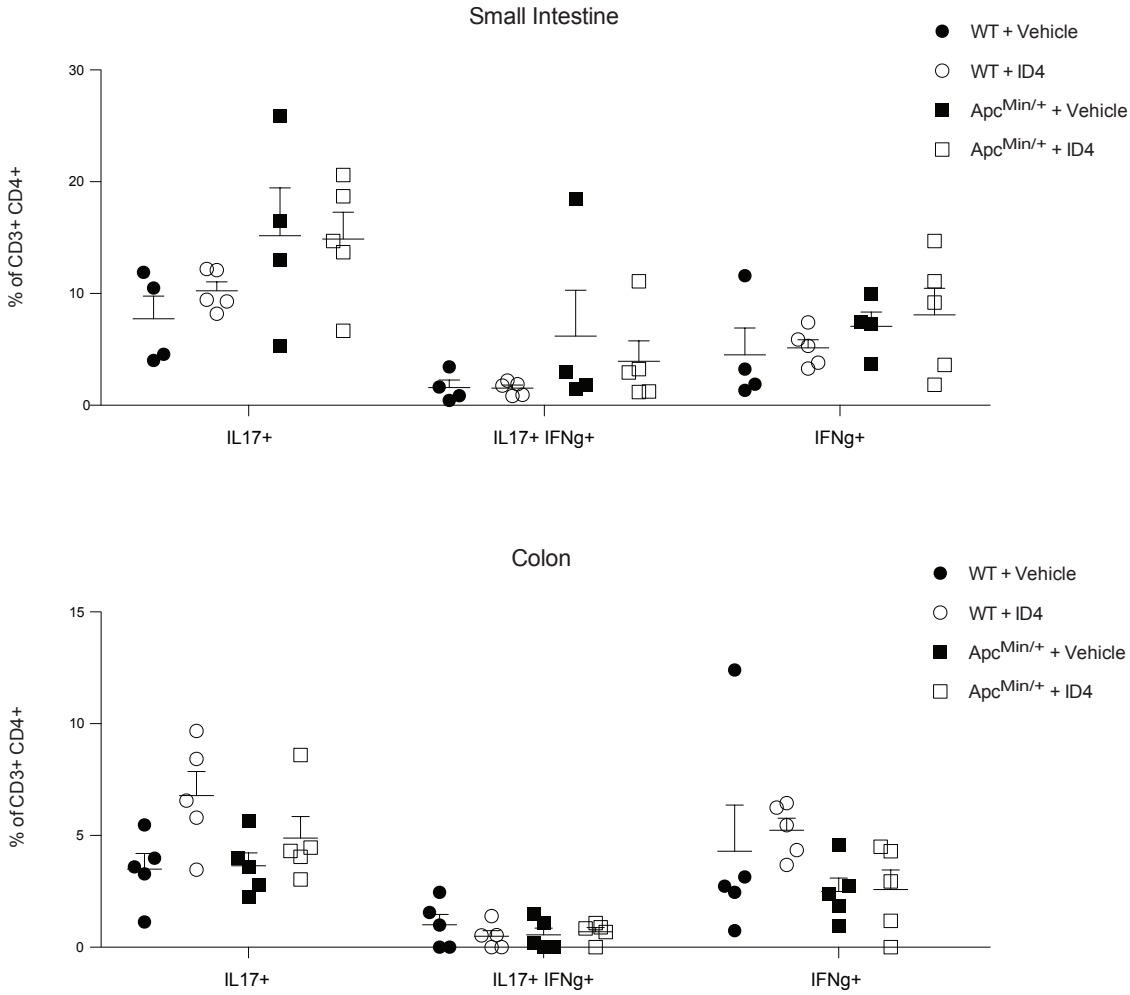

Figure 4-41 IL17 and IFNγ producing T helper cells in the small intestine and colon of WT and $Apc^{Min/+}$ mice treated with vehicle or *Clostridium* ID4. WT and $Apc^{Min/+}$ mice received either vehicle or *C.* ID4 (ID4) from 6 to 12 weeks of age. At 12 weeks of age cells were isolated from the small intestinal and colonic lamina propria and restimulated *in vitro* with PMA and ionomycin in the presence of GolgiStop. The percentage of IL17+ and IFNγ+ cells (within the CD3+ CD4+ population) evaluated by flow cytometry. Graphs show mean ± SEM. n=5.

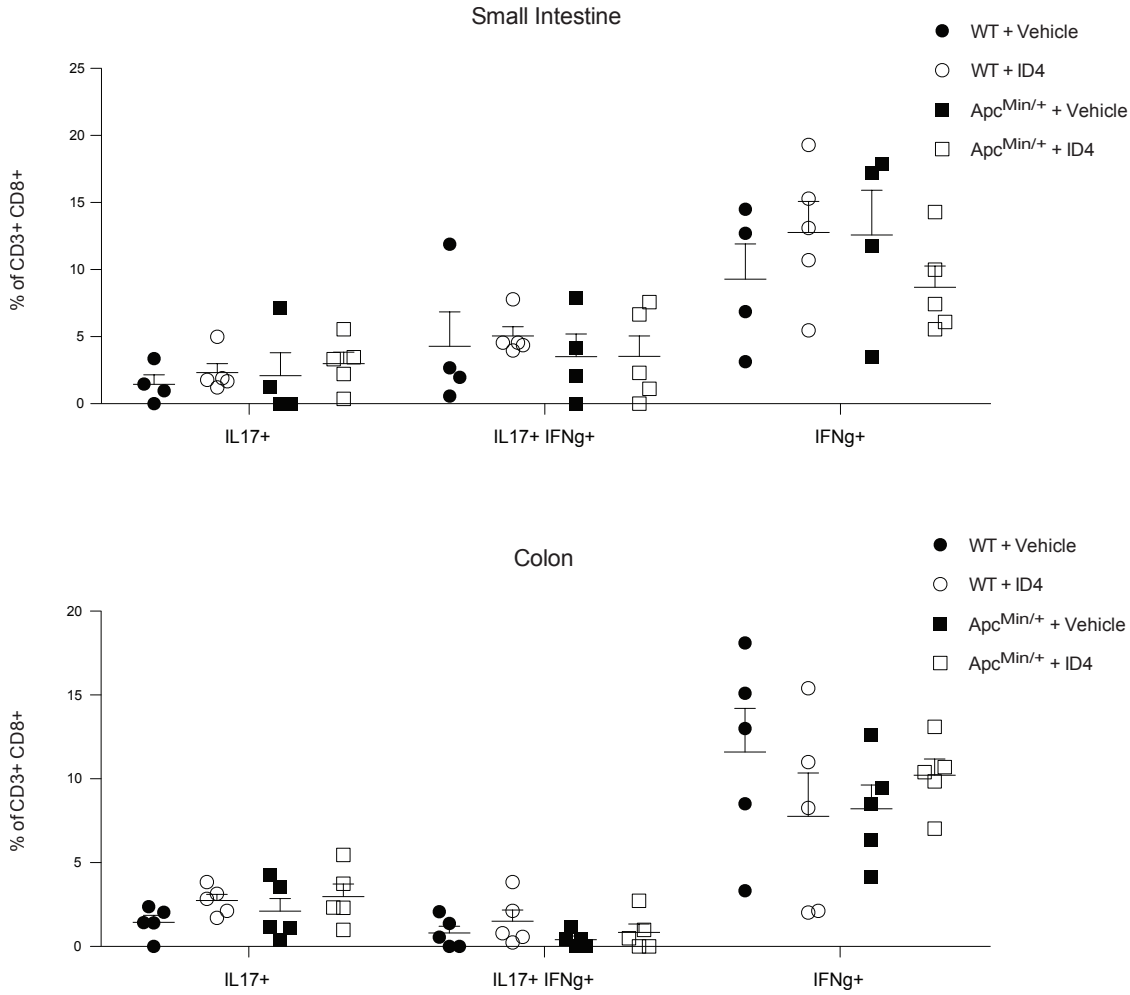

Figure 4-42 IL17 and IFNγ producing CD8+ T cells in the small intestine and colon of WT and Apc$^{Min/+}$ mice treated with vehicle or *Clostridium* ID4. WT and Apc$^{Min/+}$ mice received either vehicle or *C*. ID4 (ID4) from 6 to 12 weeks of age. At 12 weeks of age cells were isolated from the small intestinal and colonic lamina propria and restimulated *in vitro* with PMA and ionomycin in the presence of GolgiStop. The percentage of IL17+ and IFNγ+ cells (within the CD3+ CD8+ population) evaluated by flow cytometry. Graphs show mean ± SEM. n=5.

We did perform correlation analysis to highlight relations among the different parameters evaluated in the experiment. Interestingly, we found that *C*. ID4 abundance in the faeces at 12 weeks inversely correlates with the tumor number in the small intestine, with mice having very low *C*. ID4 levels displaying the highest number of polyps in the SI (Figure 4-43). Moreover fecal *C*. ID4 levels at the 12th week of age is also positively correlated with Tregs abundance in the SI (Figure 4-43). Of note, both these correlations were found in the vehicle treated Apc$^{Min/+}$ group, pointing to a physiological significance of these data in our tumorigenesis model.

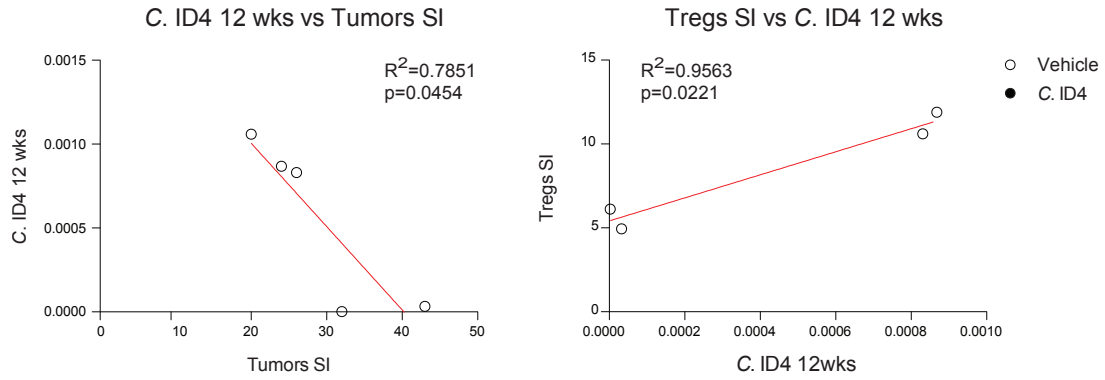

Figure 4-43 Correlation between *Clostridium* ID4 abundance at 12 weeks and tumor number and T regulatory cells in the small intestine. *C.* ID4 adundance in the faeces at 12 weeks of age was correlated with tumor multiplicity in the small intestine and with the percentage of Treg cells in the small intestine. Both correlations were done for the vehicle treated $Apc^{Min/+}$ group. R^2 and p value for each correlation are shown.

4.7 miRNA profile is altered in $Apc^{Min/+}$ intestine

The mechanism through which the expression of the wild type *apc* allele is lost leading to polyposis in FAP and in $Apc^{Min/+}$ mice is classically described as loss of heterozygosity, meaning the loss of the entire genetic locus comprising the allele of interest. In the last years this hypothesis has been challenged (Amos-Landgraf et al., 2012), arguing that epigenetic mechanisms might be involved in the loss of expression of the normal allele. Since we observe that tumor bearing mice have a different microbiota compared to WT mice already at young age and given that bacteria are potent modulators of miRNA expression (Takahashi, 2014; Yang et al., 2013)(Saccheri F., unpublished) we investigated the possibility of miRNA being involved in the regulation of the *apc* pathway. We performed miRNA profiling of single crypts isolated by laser-assisted microdissection from wild type ilei (WT) and from normal (N) and dysplastic (D) crypts of $Apc^{Min/+}$ mice (Figure 4-44) and compared miRNA expression levels of WT vs. N crypts and D vs. N crypts applying a 10-fold change cut-off. We found that in $Apc^{Min/+}$ normal crypts 17 miRNAs were up-regulated while 35 were down-regulated compared to wild type ones,

while in dysplastic crypts 34 miRNAs were up-regulated and 17 were down-regulated (Figure 4-44).

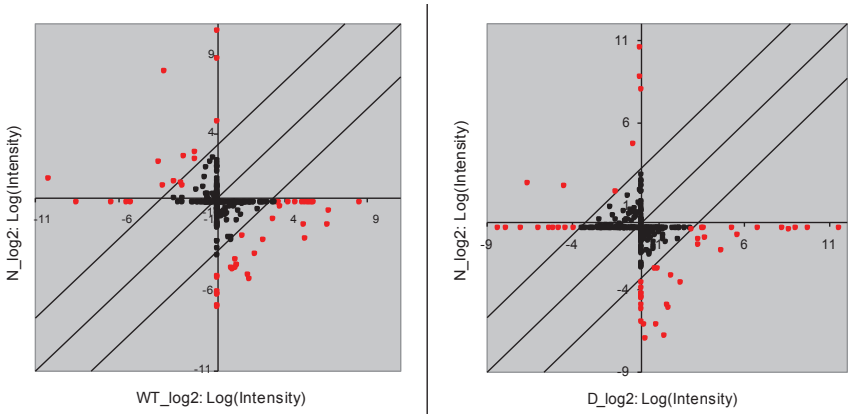

Figure 4-44 miRNA expression is deregulated in Apc$^{Min/+}$ intestines. RNA was extracted from single crypts isolated with laser-assisted microdissection from wild type mice (WT) and from normal (N) and dysplastic (D) crypts of Apc$^{Min/+}$ mice at 16 weeks of age. miRNA expression in WT vs. N and D vs. N is shown. A cut-off of 10-fold was used.

Studies in the human system have demonstrated that hsa-miR-135a/b are involved in the regulation of human *apc* gene (Nagel et al., 2008). Interestingly we observed that mmu-miR-135a/b were upregulated in dysplastic crypts of Apc$^{Min/+}$ mice (Figure 4-45). However an analysis of the predicted miRNA binding sites in the 3' UTR of murine *apc* gene using TargetScanMouse program (release 5.2) did not highlight putative mmu-miR-135a/b binding sites. However, among the miRNAs that were differentially expressed in normal or dysplastic Apc$^{Min/+}$ crypts some had as putative targets molecular components of the Wnt pathway, such as frizzled receptors and WNT proteins.

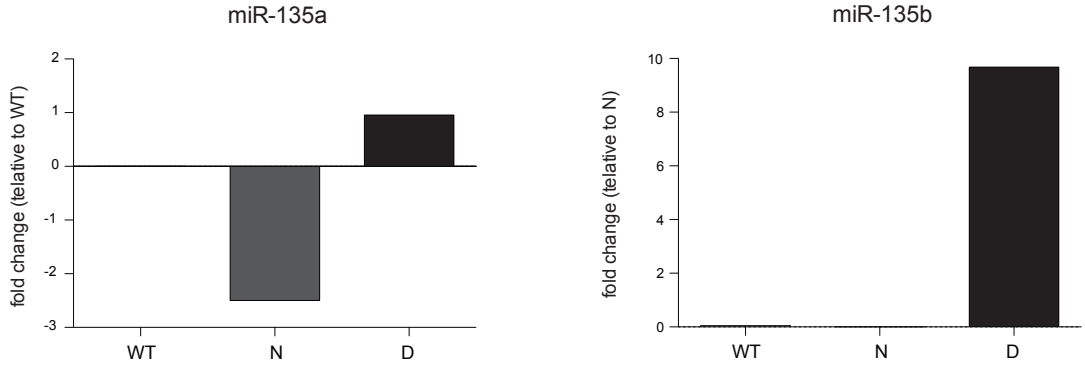

Figure 4-45 mmu-miR-135a/b are upregulated in Apc$^{Min/+}$ dysplastic crypts. RNA was extracted from single crypts isolated with laser-assisted microdissection from wild type mice (WT) and from normal (N) and dysplastic (D) crypts of Apc$^{Min/+}$ mice at 16 weeks of age. Expression levels of miR-135a (left) and miR-135b (right) are shown as fold change relative to WT and N respectively.

Preliminary data produced in our laboratory demonstrate that bacterial stimuli, namely *Salmonella*, can modulate mi-RNA expression in the B16 murine melanoma cell line (Saccheri F., unpublished). Having observed both a dysbiosis and deregulation in mi-RNA expression in the Apc$^{Min/+}$ model we addressed whether bacterial stimuli could be involved in the modulation of *apc* gene expression (Figure 4-46). We stimulated WT or Apc$^{Min/+}$ colonic mucosa using an *ex-vivo* organ culture protocol developed in our laboratory (Tsilingiri et al., 2012; Tsilingiri et al., 2013) that allows polarized stimulation of the intestinal mucosa. Colonic mucosa was stimulated with either non-invasive or invasive *Salmonella* strains. However we did not observe any changes in *apc* gene expression in the WT or Apc$^{Min/+}$ colonic tissue stimulated with either *Salmonella* strains (Figure 4-46). We stimulated the colonic mucosa also with *E. coli* as to mimic stimulation with a member of the endogenous microbiota. However stimulation of WT and Apc$^{Min/+}$ mucosa with either non-invasive or invasive *E. coli* strains did not cause major alterations in *apc* expression (Figure 4-46).

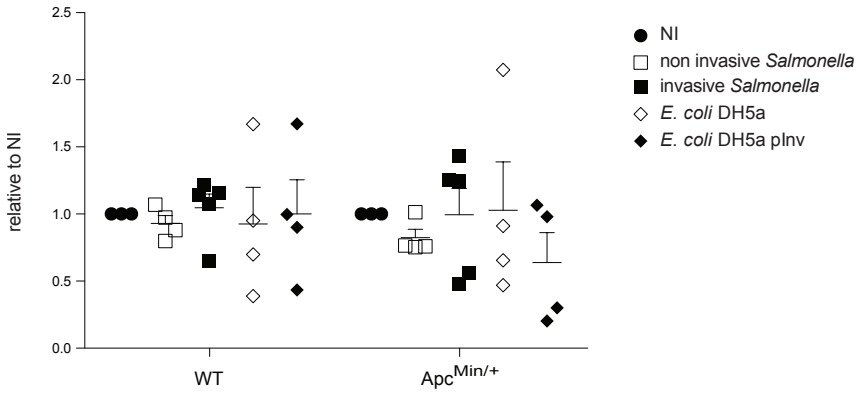

Figure 4-46 Bacterial stimulation does not alter *apc* gene expression in WT and Apc$^{Min/+}$ colon. WT and Apc$^{Min/+}$ colonic mucosa was stimulated for 2 hours with 10^2 CFUs of non-invasive or invasive *Salmonella* and non invasive or invasive *E. coli* (*E. coli* DH5a and *E. coli* DH5a pInv). After 24 hours incubation in 100% O_2 atmosphere apc gene expression was evaluated by RT-qPCR assay. Apc

expression level was normalized to Rpl32 and expressed as fold increase relative to not infected sample (NI). Graphs show mean ± SEM. n=4/5.

	4 weeks			8 weeks			12 weeks			16 weeks		
Genus	**WT**	**Apc**	**p value**	**WT**	**Apc**	**p value**	**WT**	**Apc**	**p value**	**WT**	**Apc**	**p value**
Lactobacillus	70,39	79,75	0,0590	39,84	49,94	0,3554	40,12	62,01	0,0243	40,01	28,55	0,3398
Clostridium	13,76	8,93	0,1438	31,13	20,97	0,3540	45,01	13,95	0,0010	36,38	46,21	0,3281
Mucispirillum	4,66	1,86	0,1524	3,89	2,46	0,5634	1,92	6,77	0,2232	3,40	3,39	0,9993
Bacteroides	2,54	2,01	0,5924	5,02	8,52	0,3746	1,43	4,07	0,0841	1,71	6,68	0,0322
Alistipes	2,75	2,19	0,5102	10,55	8,07	0,2593	4,96	4,82	0,8848	6,78	7,05	0,9105
Candidatus Arthromitus	1,59	1,81	0,7571	0,19	0,60	0,0671	0,02	0,11	0,2402	0,00	0,55	0,1299
Ruminococcus	0,64	0,24	0,0945	3,90	2,58	0,2676	2,05	1,71	0,5845	2,68	1,84	0,2573
Eubacterium	0,56	0,94	0,4919	1,40	1,47	0,9284	1,05	2,77	0,4279	1,84	1,05	0,5153

Appendix 1 Genus abundance in WT and Apc$^{Min/+}$ mice. The table shows the relative abundance of the principal genera in WT and Apc$^{Min/+}$ metagenomic data at 4, 8, 12 and 16 weeks of age. Abundance is shown as percentage. The p value for the comparison WT vs. Apc$^{Min/+}$ within the same time point is shown. Red values are p<0,05.

5 DISCUSSION

The microbiota is the ensemble of the microorganisms living in symbiosis with the human body. The study of this complex community and of its interactions with the host has in recent years attracted the attention of the scientific community and the interplay between the host and its microbiota has been demonstrated to be so mutualistic that the communion of the two has been described as a superorganism (Eberl, 2010).

The stage on which the interactions between the host and the intestinal microbial community do take place is the intestinal barrier (Hooper and Macpherson, 2010). A fundamental component of this barrier is the mucus layer that acts both as a niche to commensal microorganisms and as a mechanism of defence against invading pathogens (McGuckin et al., 2011). Alterations in both the mucus and the intestinal microbiota have been described in numerous pathological conditions, among which colorectal cancer (Honda and Littman, 2012; Sheng et al., 2012) (Sears and Garrett, 2014; Zhu et al., 2013). In particular studies on hCRC patients highlight alterations in the mucus barrier at the level of mucin expression and glycosylation (Sheng et al., 2011) and alterations in the intestinal microbial community, such as the increase in *Fusobacterium nucleatum* observed in the tumor tissue compared to the healthy one (Kostic et al., 2012). However, although these and other studies on hCRC patients have been fundamental in the dissection of the alterations associated with CRC, they do not allow dissection of the timing of the onset of these alterations and their cause-effect relationships. For this reason we employ here murine models of intestinal tumorigenesis to dissect how alterations in the intestinal mucus barrier and dysbiosis are interconnected during tumor progression.

5.1 The mucus barrier is altered in murine models of intestinal tumorigenesis

The mucus is a fundamental but often neglected component of the intestinal barrier. Different studies in murine models show its fundamental role in the interaction with the endogenous microflora. $Muc2^{-/-}$ and $Muc13^{-/-}$ mice are more susceptible to experimental colitis (Sheng et al., 2011; Van der Sluis et al., 2006). On the contrary, $Muc1^{-/-}$ mice are protected from colitis (Petersson et al., 2011). Interestingly $Muc2^{-/-}$ mice do also spontaneously develop colon cancer (Velcich et al., 2002). All of these studies underline the importance of a proper mucus barrier for intestinal homeostasis. As a matter of fact alterations in the mucus layer are documented in IBD and CRC (Sheng et al., 2012).

The $Apc^{Min/+}$ murine model is widely employed as model for human FAP (Moser et al., 1990). We used this model to demonstrate that in tumor-bearing mice, similarly to what happens in the human pathology, the mucus has altered properties. In particular the expression level of mucins that are normally expressed at low levels or not expressed in the intestine increases (e.g. Muc1, Muc20 and Muc5ac) while the expression of those mucins that are gut-specific (e.g. Muc3 and Muc13) decreases. These alterations are predominant in the small intestine of $Apc^{Min/+}$ mice, that is the intestinal segment most interested by the polyposis, while in the colon they are less evident. Alterations in mucin expression start arising early during tumorigenesis (e.g. 10 weeks), even though they become striking at late stages of the disease. Going deeper we observed that mucin expression is altered already at the level of single dysplastic crypts, strictly relating changes in the mucus with neoplastic transformation. Much of the alterations we observe in our murine model parallel the hCRC situation. For example the increased expression of MUC1 has from long been associated with intestinal adenocarcinomas (Ajioka et al., 1996) and MUC5AC is ectopically expressed in mucinous colorectal cancers (Park et al., 2008). High Muc20 expression levels are associated with recurrence and poor prognosis in CRC patients and its overexpression enhances malignant phenotype *in vitro* (Xiao et al., 2013). Interestingly, a recent work focusing on human small bowel cancer reported increased Muc1 and Muc5ac expression, with the

latter correlating with poor disease outcome (Shibahara et al., 2014). However, contrarily to what we do observe in our murine model, Muc13 overexpression is reported to correlate with malignancy in hCRC (Gupta et al., 2012).

Regarding aberrancies at the level of mucin post-translational modifications we did not observe differences in glycosylation as far as sulfomucin abundance is concerned. However, if on one hand we detected sulphonation signal in the colon of WT and $Apc^{Min/+}$ mice as reported in the human large bowel (Matsuo et al., 1997) we barely detected any signal in the small intestine even in WT mice. Given that the small intestine is the site most interested by tumorigenesis in the $Apc^{Min/+}$ model there could be alterations in other mucin post-traslational modifications that we did not consider in this work, such as fucosylation that has very recently been shown to regulate host-microbiota interaction upon TLR stimulation through ILC3 produced IL22 (Goto et al., 2014a; Pickard et al., 2014).

Interestingly, exogenous disruption of the mucus layer using the mucolytic N-acetyl cysteine (NAC) does not alter mucin expression in WT mice. These data concomitantly to the previously presented ones open the way to the hypothesis that *apc* mutation is needed for mucin expression alterations in the $Apc^{Min/+}$ model.

We have thus shown that the $Apc^{Min/+}$ model shows alterations at the level of mucin expression and many of these parallel what is reported for hCRC.

5.2 The intestinal barrier is altered upon tumorigenesis

Being the mucus a fundamental albeit often ignored component of the intestinal barrier we addressed whether bacterial penetrance was altered in tumor-prone mice. Barrier properties towards an enteric pathogen such as *Salmonella* typhimurium are altered in $Apc^{Min/+}$ mice, in that higher bacterial loads are retrieved from the small intestine, polyps and mLNs if compared to WT littermates. Since we observe similar results with a non-invasive *Salmonella* strain and given the comparable bacterial spreading that we observe when mice are challenged via the intravenous route we interpret these data as supporting the evidence of altered intestinal barrier properties in tumor-prone murine

models. This is in accordance with the work of Grivennikov and colleagues that show increased colonic permeability to FITC-dextran and bacterial products in a model of APC driven intestinal tumorigenesis (Grivennikov et al., 2012). In the same work the authors observe altered Muc2 and barrier protein expression in the tumor (Grivennikov et al., 2012). So neither our data nor Grivennikov and colleagues allow discrimination between the contribution of epithelial barrier or mucus layer in the alteration of the intestinal barrier properties upon tumorigenesis. However the use in this work of *Salmonella*, a bacterium that is able to interact with the mucus (McCormick et al., 1988; Nevola et al., 1987; Vimal et al., 2000) to investigate intestinal permeability could allow the detection of differences due to the diversity of the mucus barrier in tumor bearing mice. This is further supported by results obtained with the non-invasive strain that has impaired ability to invade the epithelium but is hypothetically identical in mucus-binding properties.

5.3 Mucus disruption exacerbates intestinal tumorigenesis

Since mucus alterations are documented in hCRC and here shown to occur also in a murine model we addressed the functional meaning of this by exogenously altering the mucus layer in murine models of intestinal tumorigenesis.

Many commensal microorganisms and enteric pathogens bind to mucins and express mucin-degrading enzymes that degrade the mucus barrier using it as nutrient source and allowing access to the epithelium (Derrien et al., 2010; Ouwerkerk et al., 2013). *Salmonella* is one of these microorganisms (McCormick et al., 1988; Nevola et al., 1987; Vimal et al., 2000). Hence we used it to see whether a mucus-degrading bacterium could alter tumorigenesis in the $Apc^{Min/+}$ model. We did indeed observe an increase in colonic tumorigenesis while we detected no difference in the small intestine. This can be interpreted in two ways. On one hand *Salmonella*, especially in streptomycin pretreated mice, has a preferential tropism for the terminal ileum and large intestine (Barthel et al., 2003) and as a consequence these could be the sites where it colonizes and acts most effectively. On the other hand, since polyposis penetrance is maximal in the small intestine in the $Apc^{Min/+}$ model, in this district the phenomenon could be at saturation not

allowing detection of differences at the late time point analysed (16 weeks). However, we cannot exclude that the increase of colonic tumors observed here is due to colitis inducing effect of *Salmonella*, similarly to what is observed in $Apc^{Min/+}$ mice infected with *Citrobacter rodentium* (Newman et al., 2001). Of note, also in the case of *C. rodentium* infected $Apc^{Min/+}$ mice the increase in polyposis is observed in the colon and not in the small intestine (Newman et al., 2001).

The second approach employed here to disrupt the mucus barrier is through the use of the mucolytic agent N-acetyl cysteine (NAC). We analysed in this case the AOM/DSS model of chemically induced colitis associated colonic carcinogenesis coupled or not with NAC treatment. Treatment with the mucolytic agent exacerbated tumorigenesis increasing the number of adenocarcinoma lesions in the colon. DSS is a colitis inducing agent that causes epithelial disruption (Perse and Cerar, 2012). For this reason it is used as a promoter agent in different intestinal tumorigenesis models, such as AOM/DSS (Neufert et al., 2007), and has been shown to induce colonic tumorigenesis in the genetically driven $Apc^{Min/+}$ model (Tanaka et al., 2006). However we show here that treatment with NAC, that does not induce epithelial damage nor inflammation, has an effect in increasing the number of adenoma and carcinoma lesions found in the colon underlining the role of mucus disruption in the exacerbation of intestinal tumorigenesis.

5.4 Dysbiosis arises early during tumor progression

The mucus is fundamental in maintaining homeostasis between the host and the microbiota in that it serves as a niche for commensals that colonize it (Johansson et al., 2008) and get nutrients from it (Derrien et al., 2010). Alterations in the mucus as the ones that we do observe in the $Apc^{Min/+}$ model could then lead to alterations in the microbial community inhabiting it. The alteration of the microbial community homing a specific niche is termed dysbiosis. This phenomenon is well documented in hCRC and numerous efforts are trying to link this to CRC pathogenesis itself (Arthur and Jobin, 2013; Zhu et al., 2013). However, only very few cases functionally linking microbial alterations to tumorigenesis have been reported to date. Seminal works by Kostic and

colleagues identified enrichment in *Fusobacterium nucleatum* in the tumor samples compared to matched controls and further proved the ability of this particular species to induce both small intestinal and colonic tumorigenesis in the $Apc^{Min/+}$ model (Kostic et al., 2013; Kostic et al., 2012).

We analysed dysbiosis occurrence in the $Apc^{Min/+}$ model to address the time frame interested by its onset, something that is not possible to dissect in the human system. We performed metagenomic analysis in the faeces of wild type and $Apc^{Min/+}$ mice at different ages (and hence stages of tumor development) to see whether we do observe a dysbiosis in our murine model and when it is arising. Interestingly we found that the microbiota composition of $Apc^{Min/+}$ mice is altered already at 4 weeks, an age at which the mice are completely tumor-free and are still cohoused with WT littermates. In particular, we observed an expansion of the *Lactobacillus* genus in $Apc^{Min/+}$ mice at 4, 8 and 12 weeks of age, concomitantly with a contraction in the *Clostridium* genus. At 16 weeks this trend was inverted. This is possibly explained by the emergence of secondary symptoms of the disease, such as intestinal bleeding, that may alter the gut microenvironment. The finding that Clostridia are underrepresented in $Apc^{Min/+}$ mice already at young age is of particular interest since the presence of these bacteria has been associated to the differentiation of functional T regulatory cells in the intestine (Atarashi et al., 2013; Atarashi et al., 2011a). Absence of Clostridia could explain the aberrant Treg functionality observed by Gounaris et al. in the $Apc^{\Delta 468/+}$ murine model (Gounaris et al., 2009).

Hence, differential abundance of bacterial groups in tumor bearing individuals could arise in early phases of tumorigenesis and alter intestinal homeostasis in a way that favours or not the process. Studies on hCRC samples do not allow dissection of the cause-effect relationship occurring between tumor formation and dysbiosis onset. Our results only partially answer to this question in that we see alterations at the genus level already at 4 weeks, an age at which $Apc^{Min/+}$ mice do not yet have polyps and are still cohoused with WT littermates. However these differences become significant only at later time points making rather speculative the statement that dysbiosis precedes

tumorigenesis. What we do observe is that dysbiosis is an early event paralleling tumor progression and this renders particularly intriguing the study of the interplay between the microbiota and the developing tumor and how each of the two can influence the other.

5.5 *Clostridium* ID4 is depleted in Apc$^{Min/+}$ mice

Metagenomic analysis of Apc$^{Min/+}$ mice over the course of tumor progression highlights that at the OTU level *Clostridium* ID4 is significantly depleted in Apc$^{Min/+}$ faeces at 8 and 12 weeks of age compared to WT littermates. Interestingly *Clostridium* ID4 is reduced not only in the faeces, but also in the microbial communities inhabiting the mucus layer of the small intestine and of the colon of Apc$^{Min/+}$ mice. The finding that *Clostridium* ID4 is underrepresented in tumor bearing mice is of particular relevance since *Clostridia* species belonging to clusters IV, XIVa and XVII isolated from human faeces are involved in the differentiation of Tregs in the intestine (Atarashi et al., 2013; Atarashi et al., 2011a). *Clostridium* ID4 does not belong to these clusters but could be species-specific for the mouse. In addition to this another Clostridia-related species, such as SFB, is involved in the maturation of Th17 cells, another immune cell population that plays a fundamental role in intestinal homeostasis and in the pathogenesis of intestinal tumors (Chae et al., 2010; Goto et al., 2014b; Ivanov et al., 2009; Ivanov et al., 2008). Thus the study of why *C*. ID4 is lost during tumorigenesis and which are the effects of this phenomenon on tumorigenesis could provide a functional link between an endogenous species and tumor progression.

5.6 *Clostridium* ID4 administration does not alter tumor multiplicity nor immune cell populations in Apc$^{Min/+}$ mice

Kostic and colleagues have reported an increase in *Fusobacterium nucleatum* in tumor samples compared to matched controls and have demonstrated that this species harbour tumorigenic properties by exogenously administering it in the Apc$^{Min/+}$ model (Kostic et al., 2013; Kostic et al., 2012). Having observed depletion of *C*. ID4 in Apc$^{Min/+}$ mice we performed a similar experiment of exogenous administration of *C*. ID4 to

$Apc^{Min/+}$ mice to address whether exogenous administration of a species that, contrarily to *F. nucleatum*, gets lost during tumorigenesis could attenuate the penetrance of the disease. Even though there are reports on the use of probiotics in the clinics for treatment of CRC (Kahouli et al., 2013) this would be to our knowledge the first report of an endogenous species having a protective role towards tumor development.

We orally administered *C*. ID4 to $Apc^{Min/+}$ and WT littermate mice from 6 to 12 weeks of age. Interestingly, although treated $Apc^{Min/+}$ mice have higher *C*. ID4 level compared to vehicle treated $Apc^{Min/+}$ they did not reach the levels retrieved from WT mice. This could mean that although exogenously administered, *C*. ID4 does not find its proper niche in $Apc^{Min/+}$ mice possibly because of the alterations that we observe in the mucus composition. *C*. ID4 treated $Apc^{Min/+}$ mice displayed a slight reduction in small intestinal tumor multiplicity. However *C*. ID4 treatment did not result in alterations in the main T cell populations inhabiting the gut in WT and $Apc^{Min/+}$ mice and whose differentiation has been linked to the microbiota (Tregs, Th17, Th1) although causing an increase in colonic Tregs and small intestinal Th17 in gnotobiotic monocolonized mice. Interestingly when we analysed individual mice we observed that *C*. ID4 abundance in the faeces at 12 weeks of age inversely correlated with tumor multiplicity and positively correlated with Tregs abundance in the small intestine.

Thus, *Clostridium* ID4 administration at the dosage and timing employed does not alter tumor multiplicity nor immune cell abundance in $Apc^{Min/+}$ mice. However, the results obtained with correlation analysis suggest that increasing the number of mice and the administered dose of *C*. ID4 could highlight subtle differences. Hence we are currently analysing the effect of anticipating *C*. ID4 treatment to weaning age (4 weeks of age) and of a closer administration schedule (3 instead of 2 administrations per week).

5.7 miRNA involvement in apc expression in the $Apc^{Min/+}$ murine model

In the human FAP the mechanism through which the normal *apc* allele is lost is described as loss of heterozygosity, a phenomenon that entails the loss of the genomic

region comprising the *apc* locus (Markowitz and Bertagnolli, 2009). However recent studies in rat models have challenged this view demonstrating that in some cases there is maintenance of heterozygosity at the genomic level but loss of expression of the WT allele (Amos-Landgraf et al., 2012). This paves the way to the contribution of epigenetic mechanisms in tumor initiation. Bacteria have recently been proposed to modulate the epigenome (Takahashi, 2014; Yang et al., 2013). In particular several pathogens alter host miRNA response and *Salmonella* is one of these (Maudet et al., 2014). Since we observe a higher intestinal permeability in the $Apc^{Min/+}$ model and increased host-bacteria interaction and we have observed that the bacterium *Salmonella* typhimurium alters miRNA expression in the murine melanoma cell line B16 (Saccheri F., unpublished) we investigated possible bacteria mediated miRNA control of *apc* expression.

We performed miRNA profiling in single crypts from wild-type mice, normal and dysplastic crypts from $Apc^{Min/+}$ mice and observed profound alterations in both normal and dysplastic crypts isolated from $Apc^{Min/+}$ ilei. In the human miR-135a/b is overexpressed in CRC tissue and detected in patient stools when compared to healthy controls (Koga et al., 2010) and these miRNAs regulate the expression of human *apc* gene (Nagel et al., 2008). Similarly we observed that both miR-135a and b are overexpressed in dysplastic crypts. Given that the human and murine *apc* mRNA sequences have 87% identity, it is possible that murine miR-135a and b regulate murine *apc* transcript in a similar way to what happens in the human system even if murine apc has not been validated to date as miR-135a/b target. However, among the putative binding sites of these miRNAs there are promoters of other components of the Wnt signalling pathway, such as Wnt3, suggesting possible alternative ways of regulation. In addition to miR-135a/b other miRNAs that we observe to be differentially expressed in normal or dysplastic $Apc^{Min/+}$ crypts could putatively regulate components of the Wnt pathway, such as different frizzled receptors and WNT proteins.

Finally we investigated whether bacterial stimuli could alter the expression of murine *apc* in colonic explants derived from both WT and $Apc^{Min/+}$ mice. We stimulated colonic explants with a model of enteric pathogen, *Salmonella*, either invasive or not and a

model of intestinal commensal, *Escherichia coli*, either invasive or not. We did not observe alterations in *apc* gene expression in the WT or $Apc^{Min/+}$ tissue under the different conditions analysed.

5.8 Conclusions and future directions

In conclusion we found that the intestinal barrier is altered in tumor bearing mice possibly due to alterations in the mucus layer. Even though the cause-effect relationships of this do not emerge clearly from the present work alterations in the mucus could lead to the establishment of a dysbiosis. We further hypothesised that bacterial species that are underrepresented in tumor bearing subjects could be protective in the context of tumor progression. We tested here *Clostridium* ID4 that we found to be underrepresented in tumor bearing mice if compared to WT littermates. However *C*. ID4 does not induce tumor regression at the dosage and administration schedule employed. Finally, we addressed whether dysbiosis could induce alteration in miRNA-mediated regulation of *apc* gene expression. Even though we observe a markedly different miRNA expression pattern in tumor bearing intestine compared to WT the bacterial stimuli that we addressed did not produce significant modulation of *apc* gene expression.

Current and future work will be aimed at further analysing the contribution of *Clostridium* ID4 to tumor progression. In particular we are analysing the effect of a closer *C*. ID4 administration schedule to confirm the mild reduction in tumor multiplicity that we do observe. We will then analyse the mechanism that involve *Clostridium* ID4 in the tumorigenesis process, envisaging these main possibilities:

1. *Clostridium* ID4 might have an effect on the immune system. In particular it could expand the Treg cell population as observed for other Clostridia species (Atarashi et al., 2013; Atarashi et al., 2011a) and this could ameliorate the intestinal inflammatory status and retard tumor progression. In the *C*. ID4 *in vivo* administration experiment already performed we did not observe alterations in Treg abundance but this is possibly due to the lower bacterial doses employed;

2. The effect observed on tumorigenesis might not be due to *Clostridium* ID4 *per se* but this bacterium could re-establish a balance in the intestinal microbial community that is lost upon tumorigenesis and that is beneficial to the host and slows down tumor progression. We will address this point by performing metagenomic sequencing in $Apc^{Min/+}$ mice treated or not with *C*. ID4;
3. *Clostridium* ID4 could have an effect on tumorigenesis through production of metabolites that have antitumorigenic effect, such as SCFAs or bile acids (Modica et al., 2008; Sears and Garrett, 2014; Smith et al., 2010).

If we will not observe the expected difference in tumor multiplicity upon *C*. ID4 administration we could hypothesize that this is due to an inability of the bacterium of colonizing the intestinal microenvironment. This might be due to the fact that mucus is altered in $Apc^{Min/+}$ mice and that *C*. ID4 is detected also at the level of the mucus associated bacterial community. Possible scenarios will then entail alterations of the mucus coupled with *C*. ID4 treatment.

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